3. The microsome subfractions were analysed for protein, ribonucleic acid, deoxyribonucleic acid and lipid-soluble phosphorus. Applying usual procedures for the separation of protein and nucleic acid, the ribonucleic acid of several subfractions was found to be abnormally unstable in perchloric acid. Several of the subfractions were examined in the ultracentrifuge and by electrophoresis.

4. The results have been compared with those obtained by other methods of fractionation of the microsomes, and the possible role that ribonucleic acid may play in protein synthesis is discussed.

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The Breakdown of Adenosine Triphosphate Accompanying Cholic Acid Activation by Guinea-Pig Liver Microsomes

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It has previously been shown that microsomal preparations from guinea-pig liver form cholyl-hydroxamic acid from cholic acid in the presence of adenosine triphosphate (ATP), coenzyme A (CoA) and high concentrations of hydroxylamine (Elliott, 1955, 1956a). When taurine at substrate levels is added to the same system in place of hydroxylamine, taurocholic acid is formed and this reaction is also dependent on the presence of ATP and CoA (Elliott, 1956b). Bremer (1956) and Siperstein & Murray (1956) have independently reported the synthesis of taurocholic acid by microsome preparations. These observations indicate that cholyl-CoA formation is an intermediate step in taurocholic acid synthesis. The enzyme system bringing about the synthesis of cholyl-CoA will subsequently be referred to as the cholic acid-activating system.

The present paper is concerned with the breakdown of ATP which accompanies the activation of cholic acid by the cholic acid-activating system of guinea-pig liver microsomes. It is shown that adenosine 5'-phosphate (AMP) and inorganic pyrophosphate are formed. Some properties of the microsomal pyrophosphatase which complicated the investigation are also described.

EXPERIMENTAL

Hydroxylamine. Solutions of low KCl content were prepared by the method of Beinert *et al.* (1953). Norit A was kindly supplied by Dr D. E. Hughes. The inorganic phosphate present in the charcoal was removed by boiling in *n*-HCl for 15 min. and filtering with suction. The material was washed with water until the washings were neutral and dried at 105°.

ATP. This was obtained as the crystalline disodium salt (Schwarz Laboratories Inc.); solutions were adjusted to pH 7.4 with KOH.

Glyoxaline buffer. This was prepared by adjusting the pH of solutions of the free base with HCl. Measurements of pH were made at 37°.

Other preparations were as described previously (Elliott, 1956a, b).

Estimation of inorganic pyrophosphate. Two methods were used for this: first, a modification of the direct colorimetric method of Flynn, Jones & Lipmann (1954), and secondly, the determination of 10 min. acid-hydrolysable phosphate after removing nucleotides with charcoal (non-nucleotide P_{10}).

Colorimetric method

Samples (2.0 ml.) of incubation mixtures were pipetted into 2.0 ml. of 10% trichloroacetic acid and the precipitates removed by centrifuging. The supernatants were poured into centrifuge tubes and 200 mg. of washed Norit A was stirred into each tube. After 15 min. with continuous agitation, the charcoal was centrifuged off and the supernatants were poured into clean tubes and again centrifuged. Three successive centrifugings were needed to remove all the charcoal. Samples (0.5 ml.) of the final supernatants were used for the estimation of inorganic phosphate (P_i) and similar samples for the separate determination of P_i + pyrophosphate. For the latter a modification of the method of Flynn *et al.* (1954) was employed. These authors showed that Versene (ethylenediaminetetraacetic acid), present initially, caused a reduction in colour development. Use was made of this to stop the colour development at the appropriate time and so avoid the necessity of attempting to reach extinctions in solutions whose colour was rapidly increasing. After standing for 90 min., 1.0 ml. of 0.1 M ethylenediaminetetraacetic acid (EDTA) (as the sodium salt, pH 7.2) was added to each tube. Fig. 1 shows that this at once stops further development of colour and a very slow decrease then takes place. The extinctions were measured within 10 min. of adding EDTA. Internal standards of pyrophosphate were added to the unknowns.

Fig. 1. Effect of EDTA on colour development in the pyrophosphate determination. Experimental details are described in the text. The arrow shows the point at which EDTA was added. Values are corrected for the dilution due to this addition.

Estimation of non-nucleotide P_{10}

Samples (0.5 ml.) of the supernatants from the charcoal treatment were mixed with 0.5 ml. of 2N-HCl and heated at 100° for 10 min. and the total inorganic orthophosphate was estimated. After charcoal treatment it was found that there were no ultraviolet-light-absorbing spots on chromatograms of the supernatant fractions, showing that the phosphate formed on hydrolysis (P_{10}) was non-nucleotide in origin.

Inorganic orthophosphate. Estimations were by the method of Fiske & Subbarow (1925).

AMP. This was estimated chromatographically by the method of Krebs & Hems (1953). Samples (0.05 ml.) of the supernatants before charcoal treatment were applied to the paper.

Estimation of cholyhydroxamic acid. This was carried out as described previously (Elliott, 1956a), except that the standard curve from which values were taken was made by adding the standard synthetic cholyhydroxamic acid solution to 'control' incubation mixtures which did not contain cholic acid. The solutions were then treated in the same way as the experimental incubation systems. The curve obtained is thus corrected for the loss of hydroxamic acid in the protein precipitate; this amounts to 8.6% of the total.

Chromatography of inorganic pyrophosphate. Inorganic pyrophosphate was identified chromatographically in the charcoal-treated solutions. Samples (0.05 ml.) were spotted onto Whatman no. 1 papers washed with EDTA (Eggleston & Hems, 1952) and ascending chromatograms run for 18 hr. in a solvent consisting of diisopropyl ether (90 parts) and 90% formic acid (80 parts). Inorganic orthophosphate and inorganic pyrophosphate had R_F values of approximately 0.7 and 0.4 respectively when applied to paper on the same spot as a sample of the incubation mixtures. Pyrophosphate ran considerably slower under these conditions than when it was chromatographed by itself in the absence of incubation mixture.

RESULTS

Appearance of inorganic orthophosphate at low Mg^{2+} ion concentration. When the conditions of incubation previously found to be optimum for cholyhydroxamic acid synthesis (Elliott, 1956a) were used, inorganic orthophosphate was liberated from ATP. In the absence of cholic acid the amount formed was greatly reduced (Table 1). It can be seen that the phosphate release, above that due to ATPase activity, which was caused by the addition of cholic acid was equivalent to the amount of cholyhydroxamic acid formed. The apparent stoichiometry between these two might be an artifact resulting from the partial hydrolysis of pyrophosphate, as indeed proved to be the case. The action of the microsome suspension on inorganic pyrophosphate was therefore investigated. In addition, Table 1 also shows that the omission of hydroxylamine, surprisingly, did not result in a diminished phosphate release, but rather that a slight stimulation occurred. This is discussed later.

Some properties of microsomal pyrophosphatase

Preliminary experiments showed that the microsome suspension hydrolysed added pyrophosphate and that this activity was reduced by the addition of KF. However, when tests were carried out for pyrophosphatase activity under the same conditions (except for the omission of ATP) as are used for the cholic acid-activating system, it was found that the rate of hydrolysis of pyrophosphate was not sufficient to account for the orthophosphate released during the synthesis of cholyl-CoA. The tests were carried out with a concentration of pyrophosphate of $1 \mu\text{mole/ml.}$, which is the greatest amount that could be expected to accumulate if pyrophosphate was liberated during cholyl-CoA synthesis. Moreover, additional experiments showed that the pyrophosphatase activity was not inhibited by this concentration of substrate, and in fact increased hydrolysis was observed up to the highest concentration of pyrophosphate tested ($5 \mu\text{moles/ml.}$). Experiments were therefore carried out to see whether ATP stimulated microsomal pyrophosphatase, since this was the only component present in the cholic acid-activating assay system which was not present in the pyrophosphatase tests. Fig. 2 shows that a marked stimulation occurred, the activity in the presence of 0.005 M ATP being double that found with pyrophosphate alone. This is the smallest effect which has been found. With some preparations of microsomes, a fourfold increase in activity resulted from the addition of ATP. Adenosine diphosphate (ADP) and EDTA also stimulated the pyrophosphatase activity but were less effective than was ATP. AMP, orthophosphate and sulphate had no effect.

Effect of Mg^{2+} ion on microsomal pyrophosphatase in the presence and absence of fluoride. The effect of Mg^{2+} ion on the pyrophosphatase activity of microsomes is somewhat complicated. In the absence of fluoride, Mg^{2+} ion slightly increases the activity (Fig. 3), but it inhibits very strongly in the presence of fluoride (Fig. 4), complete inhibition being reached at a concentration of about 0.015 M-MgCl_2 . It is possible that the stimulatory action of ATP and ADP [which have been shown by Smith & Alberty (1956) to form complexes with Mg^{2+} ion] and of EDTA, on pyrophosphatase activity in the presence of fluoride, is due to the formation of chelates with Mg^{2+} ion which lowers its effective concentration and consequently increases pyrophosphatase activity. These findings made it possible to suppress the inorganic pyrophosphatase activity of microsomes by increasing the concentration of MgCl_2 to 0.015 M . This had no effect on the cholic acid-activating system. Under these conditions ATP no longer stimulated the pyrophosphatase as it did when the concentration of Mg^{2+} ion was 0.005 M .

Table 1. Liberation of inorganic orthophosphate at low Mg^{2+} ion concentration

Incubations were carried out at 37° for 90 min. The complete system contained 0.1 ml. of 0.5 M glyoxaline buffer, pH 7.4, at 37° ; 0.25 ml. of 0.1 M ATP; 1.25 ml. of 2 M hydroxylamine; 0.05 ml. of 0.5 M cysteine; 0.25 ml. of 0.5 M KF; 0.5 ml. of CoA (500 Lipmann units/ml.); 0.25 ml. of 0.1 M-MgCl_2 ; 0.25 ml. of 0.04 M potassium cholate; 1.0 ml. of washed microsome suspension. The volume was adjusted to 5.0 ml. with water. After incubation 2.0 ml. samples were pipetted into tubes containing 1.0 ml. of 7% (w/w) HClO_4 for hydroxamic acid determinations. Separate 2.0 ml. samples were measured into tubes containing 1.0 ml. of 10% (w/v) trichloroacetic acid, the protein was centrifuged off and inorganic orthophosphate determined in the supernatants. All values are expressed in terms of 2.0 ml. of incubation mixture.

Expt.	P_i produced (μmoles)	P_i release due to addition of cholate (1-2)	Cholyl-hydroxamic acid formed (μmoles)
1. Complete system	4.55	2.05	2.1
2. Cholate omitted	2.50	—	—
3. Hydroxylamine omitted	5.33	—	—

Fig. 2. Effect of ATP on microsomal pyrophosphatase. The complete system contained 0.1 ml. of 0.5 M glyoxaline buffer, pH 7.4, at 37° ; 0.1 ml. of 0.1 M-MgCl_2 ; 0.1 ml. of 0.5 M-KF ; 0.1 ml. of 0.1 M cysteine; 0.2 ml. of 0.01 M sodium pyrophosphate; 0.1 ml. of 0.1 M ATP; 0.3 ml. of washed microsome suspension; the volume was adjusted to 2.0 ml. with water. Incubations were carried out at 37° for 60 min. and stopped by the addition of 1.0 ml. of 10% (w/v) trichloroacetic acid; inorganic orthophosphate was estimated in the supernatants after centrifuging. All values are corrected for phosphate release which occurred in the absence of pyrophosphate. ●, With ATP; ○, no ATP.

Fig. 3. Effect of Mg^{2+} ion on microsomal pyrophosphatase in the absence of fluoride. Incubations were carried out at 37° for 30 min. The complete system contained 0.1 ml. of 0.5M glyoxaline buffer, pH 7.4, at 37° ; 0.1 ml. of 0.1M cysteine; 0.2 ml. of 0.01M sodium pyrophosphate; 0.3 ml. of washed microsome suspension. The volumes were adjusted to 2.0 ml. with water. After incubation 1.0 ml. of 10% (w/v) trichloroacetic acid was added to each tube, the protein centrifuged down and the inorganic orthophosphate determined in the supernatants. All values are corrected for the small P_i release which took place in the absence of pyrophosphate.

Table 2. *Measurement of orthophosphate release at high Mg^{2+} ion concentration*

Experimental conditions were as described in Table 1, except that 0.1 ml. of 0.3M- $MgCl_2$ was added instead of 0.1M- $MgCl_2$. Values are expressed in terms of 2.0 ml. of incubation mixture.

Expt.	P_i formed (μmoles)	Cholyl-hydroxamic acid formed (μmoles)
1. Complete	1.35	1.8
2. Cholate omitted	1.5	0.1

Formation of pyrophosphate at high Mg^{2+} ion concentration

Table 2 shows that when the level of Mg^{2+} ion is increased, the amount of orthophosphate which is released from ATP is no longer markedly stimulated by the addition of cholic acid. This phosphate release is presumably due simply to ATPase activity in the microsomes.

Table 3 shows, however, that a breakdown of ATP into AMP and inorganic pyrophosphate occurs under these conditions. The presence of pyrophosphate in Expt. 2 (Table 2) was also demonstrated by paper chromatography of the trichloroacetic acid supernatant after removal of nucleotides on charcoal. In the isopropyl ether-formic acid solvent there was a phosphate spot in the same position as that of authentic pyrophosphate added to the sample.

Fig. 4. Effect of Mg^{2+} ion on microsomal pyrophosphatase in the presence of fluoride. Experimental conditions were as described in Fig. 3, except that 0.1 ml. of 0.5M-KF was added and incubations were continued for 60 min.

Table 3. *Formation of inorganic pyrophosphate at high Mg^{2+} ion concentration*

Experimental conditions were as described in Table 2. The values are expressed in terms of 2.0 ml. of incubation mixture.

Expt.	Pyrophosphate (μmoles) determined by		Cholyl-hydroxamic acid (μmoles)	AMP (μmoles)
	Colorimetric method	P_{10} method		
1. Complete, stopped at zero time	0.25	0.43	0.25	3.1
2. Complete	4.0	4.25	2.1	6.1
3. Cholate omitted	1.72	1.98	0.35	4.07
Differences due to addition of cholate (2-3)	2.28	2.07	1.75	1.93

The results show an approximate stoichiometry between the cholate-dependent formation of pyrophosphate, of AMP and of hydroxamic acid. An exact stoichiometry between cholyhydroxamic acid and pyrophosphate production has not been obtained. In several experiments the amount of pyrophosphate liberated always exceeded that of cholyhydroxamic acid formed. Values differing as widely as 2.95 μ moles of pyrophosphate compared with 2.2 μ moles of hydroxamic acid have been obtained. This discrepancy has not been fully investigated but it may be related to the observation, described below, that a release of pyrophosphate, independent of hydroxylamine, occurs, which nevertheless requires the addition of cholic acid.

Formation of inorganic pyrophosphate in the absence of hydroxylamine. As already shown in Table 1, a release of orthophosphate occurred at low Mg^{2+} ion concentrations even in the absence of hydroxylamine. This release was found in subsequent experiments to be partly dependent on the addition of both CoA and cholic acid. High Mg^{2+} ion concentrations abolished the phosphate production but under these conditions a liberation of pyrophosphate occurs instead (Table 4). Little

inorganic pyrophosphate is produced in the absence of CoA whether cholic acid is present or not. In the presence of CoA alone the production of pyrophosphate is about one-half that in the presence of both CoA and cholic acid.

Effect of taurine and glycine on pyrophosphate formation. Although pyrophosphate release occurs in the absence of hydroxylamine, it was possible that taurine, which is a 'natural acceptor' for choly-CoA (Elliott, 1956b), and possibly also glycine, would stimulate this release. Table 5 shows that this does not in fact occur, the production of pyrophosphate (measured as non-nucleotide P_{10}) being unchanged by addition of these amino acids.

Attempt to fractionate the cholic acid-activating system

A much clearer understanding of the various results would be obtained if the enzymes involved could be separated from one another to some extent. Attempts were therefore made to solubilize and fractionate the microsomal proteins. The addition of cholic acid or treatment of suspensions of the particles with ultrasonic vibrations yielded clear-yellow solutions whose cholic acid-activating system was not sedimented by centrifuging at 100 000 g for 90 min. Attempts to fractionate these solutions with ammonium sulphate, heat treatment, etc., were not successful however. Very poor resolution into fractions was obtained and the precipitates so formed proved to be largely insoluble. In particular, no separation of inorganic pyrophosphatase activity from the cholic acid-activating system was achieved by any of the attempts. It seems probable that the 'solutions' obtained from the microsomes consisted of dispersions of very high molecular weight material rather than of true solutions of proteins.

DISCUSSION

The results show that the cholic acid-activating system of washed microsomes forms AMP and inorganic pyrophosphate from ATP in the course of converting cholic acid into choly-CoA. Although an exact stoichiometry between pyrophosphate liberation and cholyhydroxamic acid formed has not been obtained it seems clear that the synthesis of choly-CoA proceeds by the following reaction:

It should be noted that pyrophosphate release in the presence of cholic acid and CoA does not depend upon the presence of hydroxylamine. In the absence of hydroxylamine the formation of inorganic pyrophosphate is dependent on the presence of CoA and partially dependent on the presence of

Table 4. *Formation of inorganic pyrophosphate in the absence of hydroxylamine*

Experimental conditions were as described in Table 2, except that hydroxylamine was omitted. All values are expressed in terms of 2.0 ml. of incubation mixture.

Expt.	Pyrophosphate present* (μ moles)	P_i released
1. Complete	3.44	1.71
2. Complete, stopped at zero time	0.20	—
3. Cholate omitted	1.58	1.35
4. CoA omitted	0.48	1.5
5. Cholate and CoA omitted	0.22	2.0

* Measured as P_{10} , which was non-adsorbable on Norit A.

Table 5. *Effect of taurine and glycine on inorganic pyrophosphate formation*

Experimental conditions were as described in Table 2, except that hydroxylamine was omitted and 0.2 ml. of a 0.1 M solution of either taurine or glycine was added where appropriate.

Expt.	Pyrophosphate released* (μ moles)
1. No additions	2.0
2. With cholate	4.16
3. With cholate + taurine	4.20
4. With cholate + glycine	4.25

* Measured as P_{10} , which was non-adsorbable on Norit A.

cholic acid. It seems reasonable to assume that that part of the pyrophosphate release which is dependent on both cholic acid and CoA is coupled with cholyl-CoA formation. The cause of that part of the pyrophosphate formation which is dependent on the addition of CoA only has not been investigated.

Since CoA is present in catalytic amounts only, it must be possible for cholyl-CoA to be converted back into CoA by a reaction other than that with hydroxylamine. This could conceivably occur by a reaction with glycine if this were present in washed microsomes, together with an enzyme system to condense the cholyl-CoA and glycine to form glycocholic acid. Previous work (Elliott, 1956*b*) has, however, shown that no taurocholic acid synthesis occurs in the absence of added taurine, and it seems unlikely that there could be sufficient glycine present to saturate an acceptor system using this amino acid. This point has not been investigated however. More probably there is a cholyl-CoA deacylase which catalyses the hydrolysis of cholyl-CoA as follows:

Enzymes of this type have been found by Schachter & Taggart (1953), hydrolysing benzoyl-CoA, and by Gergely, Hele & Ramakrishnan (1952), hydrolysing acetyl-CoA and succinyl-CoA.

Such a system might well account for the observation that, in the presence of hydroxylamine, the pyrophosphate release is somewhat in excess of cholyhydroxamic acid formation. If the reaction of the cholyl-CoA with hydroxylamine is insufficiently rapid then some of the cholyl-CoA would be broken down by the deacylase and inorganic pyrophosphate would be produced without any corresponding production of hydroxamic acid.

The inhibition of microsomal pyrophosphatase by Mg^{2+} ion in the presence of fluoride, but not in its absence, has not been reported previously so far as the author is aware. Whether the reason for the effect is the existence of two pyrophosphatases, or the formation of an inhibitory complex involving Mg^{2+} ion and fluoride, is not known. The results suggest that, at any rate in the presence of fluoride, effects of compounds on pyrophosphate levels in experiments with microsomes should be interpreted with caution, in case the effect is an indirect one due to chelation of a metal ion and consequent change in the rate of pyrophosphate breakdown.

The activation of cholic acid occurs by a reaction analogous to those for acetic acid (Lipmann, Jones, Black & Flynn, 1952), short-chain fatty acids (Mahler, Wakil & Bock, 1953) and long-chain fatty acids (Kornberg & Pricer, 1953), involving the breakdown of ATP to AMP and inorganic pyrophosphate. The enzyme system activating long-chain-fatty acids occurs in microsomes as well as in

the soluble fraction (Kornberg & Pricer, 1953). It cannot be decided from the present work whether the cholic acid-activating system is the same as or different from the system activating long-chain fatty acids.

The reaction by which taurocholic acid is formed from cholyl-CoA is similar to that for hippuric acid formation (Schachter & Taggart, 1954), though the latter system is localized in the mitochondria and not in the microsomes. The synthesis of the peptidic bonds of glutamine (Speck, 1949; Elliott, 1951), and of the two peptide bonds of glutathione (Mandel & Bloch, 1955; Snoke, Yanari & Bloch, 1953), proceed by quite different mechanisms which do not involve CoA and result in the breakdown of ATP to ADP and inorganic orthophosphate. Among the small peptides which accumulate in reasonably large amounts in animal tissues the only ones which have not been synthesized in an *in vitro* system would now appear to be carnosine and its methylated derivative anserine.

SUMMARY

1. At low concentrations (0.005 M) of Mg^{2+} ion the synthesis of cholyl-coenzyme A by guinea-pig liver microsomes is accompanied by a release of inorganic orthophosphate from adenosine triphosphate (ATP). This release is abolished by Mg^{2+} ion at a concentration of 0.015 M.

2. The inorganic pyrophosphatase of microsomes is strongly inhibited by Mg^{2+} ion in the presence, but not in the absence, of fluoride. ATP, adenosine diphosphate and ethylenediaminetetraacetic acid can activate the enzyme, probably by chelating Mg^{2+} ion, provided that fluoride is present.

3. At a Mg^{2+} ion concentration of 0.015 M, cholyhydroxamic acid synthesis is accompanied by a release of inorganic pyrophosphate, though it has not been possible to demonstrate an exact stoichiometry between them. The release of pyrophosphate is not dependent on the presence of hydroxylamine. Possible explanations of these results are discussed.

4. The synthesis of oligopeptides in animal tissues is briefly discussed.

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The Nature of the Fluctuating Ribonucleic Acid in *Escherichia coli*

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In several species of bacteria growing cells have been shown to have much higher concentrations of ribonucleic acid (RNA) than resting cells. Apart from the work of Belosersky and his collaborators (Belosersky, 1947) there has been little investigation into the nature of the 'additional RNA' in growing cells; more attention has been paid to its possible function in the cell. The most widely accepted view, that it plays a part in the synthesis of protein, is based mainly upon the direct relationship observed between the RNA concentration and the growth rate of micro-organisms growing exponentially (Caldwell, Mackor & Hinshelwood, 1950; Price, 1952; Wade, 1952; Jeener, 1953), upon the presence of RNA in granules having the ability to synthesize proteins (Gale & Folkes, 1955a) and upon the concomitant syntheses of protein and RNA which occurs during enzyme induction (Pardee, 1954). Some experimental results, however, do not lend themselves to this theory, and there is increasing evidence that the level of RNA concentration does not reflect the rate at which a cell is synthesizing protein.

The contrasting RNA concentrations in resting cells and growing cells is more pronounced in some species than in others. In *Escherichia coli* the concentration of RNA in rapidly growing cells is approximately twice the concentration in resting cells, and in certain other species more than fivefold differences in concentration have been observed. The abundance of the 'additional RNA' in fast-growing bacteria and the conflicting evidence presented for its connexion with protein synthesis prompted the closer examination of its nature and function in *Esch. coli*.

It was important in the first instance to establish whether or not the increased RNA concentration which develops in resting cells during the early stages (division lag) of their growth in fresh medium is due to a general increase in the concentration of all RNA constituents or to only a fraction of these. To this end the chemical compositions, the solubilities, the responses to various methods of extraction and the sedimentation behaviours of ribonucleic acids in resting and in dividing cells of *Esch. coli* were compared. Concurrently with these experiments the effects of various extractions and fractionations upon the basophilia of the cells were observed in order to examine the relationship between the 'additional RNA' and the high basophilia of dividing cells. This report describes these experiments and suggests an alternative function for 'additional RNA' based upon our results and upon other published experimental data.

A preliminary note on part of this report has already been published (Wade, 1955).

MATERIALS AND METHODS

Organisms and cultural conditions. Comparisons between the RNA concentrations in resting and in dividing cells were made on *Aerobacter aerogenes* (NCTC 418), *Bacillus anthracis* (Weybridge), *Bacillus cereus* (NCTC 8035), *Chromobacterium prodigiosum* (laboratory strain), *Chromobacterium violaceum* (NCTC 7917), *Clostridium welchii* (SR 12), *Corynebacterium hoffmannii* (NCTC 8338), *Eberthella typhosa* (Ty 479, a purine-requiring avirulent mutant kindly supplied by Dr T. W. Burrows), *Escherichia coli* (NCTC 1100), *Pasteurella pestis* (Tjwidej), *Proteus vulgaris* (laboratory strain) and *Staphylococcus aureus* (Oxford 'H') grown on casein-casein-yeast medium (CCY) (Gladstone & Fildes, 1940) or in tryptic-meat broth (Table 1) at 37°.

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