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Biosynthesis of Polynucleotides

5. THE EFFECT OF ADENINE ON THE UTILIZATION OF GLUCOSE FOR RIBONUCLEIC ACID SYNTHESIS IN *CANDIDA UTILIS**

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During the previous work on the formation of RNA in *Candida utilis* (Barker, Hignett, Jackson & Wadsworth, 1961) it was found that addition of adenine to cultures growing exponentially in a synthetic medium containing glucose as the sole carbon source reduced the incorporation of isotopic carbon from [¹⁴C]glucose into the ribosyl residues of RNA. A preliminary report of some of the experiments has been published (Barker, Hignett, Nicholson & Thompson, 1962). The present paper records in detail the conditions under which the effect is observed.

MATERIALS AND METHODS

Analytical methods. Ribose was determined by the method of Albaum & Umbreit (1946), glucose by the iodometric method of Somogyi (1952), and phosphorus by the method of Fiske & Subbarow (1925). Concentrations of nucleotide solutions were measured spectroscopically,

appropriate molecular extinction coefficients being used (Beaven, Holiday & Johnson, 1955). Radioactivities of nucleotides and ribose were determined with an end-window counter as previously described (Barker *et al.* 1961). Radioactivity of BaCO₃ was measured with the same end-window counter and layers of BaCO₃ containing not less than 20 mg./cm.² on planchets having an area of 1 cm.². Specific activities of BaCO₃ were determined by comparison of experimental samples with a standard planchet.

Preparation of mixed ion-exchange resin column. Amberlite IR-45 (Rohm and Haas Co., Philadelphia, U.S.A.) and Zeo-Karb 225 (The Permutit Co., London) (both 200 mesh) were washed with hot ethanol to remove coloured material and were converted respectively into the OH⁻ and H⁺ forms (after repeated cycling through salt forms). The resins were mixed in quantities inversely proportional to their capacities, and a slurry of the mixture was introduced into a column (10 cm. × 1 cm.) and the liquid removed rapidly by application of pressure to avoid separation of the resins during settling.

Growth of yeast. The organisms used were *Candida utilis* strain IMI 23311, obtained from the Commonwealth Mycological Institute, and strain NCYC 321 supplied by

* Part 4: Barker, Hignett, Jackson & Wadsworth (1961).

the Brewing Research Foundation, Nutfield, Surrey. (The cultures were supplied as *Torulopsis utilis*: the name *Candida utilis* is taken as being synonymous with this; Skinner & Fletcher, 1960.) The latter strain had been maintained from a culture of strain IMI 23311 originally supplied by the Commonwealth Mycological Institute. The two strains were indistinguishable in behaviour. Both strains were maintained on nutrient agar slopes and grown in aerated glucose-salts medium at 30° as described by Barker *et al.* (1961). The organism was also maintained in a continuous culture apparatus from which exponentially growing cells were withdrawn when required. Growth was measured either with a colorimeter (Evans Electro-selenium Ltd., Harlow, Essex) with a red filter, or by measurement of extinction at 670 m μ . In some experiments, cell populations were measured in a haemocytometer after suitable dilution with 0.14M-NaCl.

Extraction and hydrolysis of sodium nucleates. Sodium nucleates were extracted from washed cells, hydrolysed by KOH, and the mixed nucleoside 2'- and 3'-phosphates were either separated by ion-exchange chromatography, as described by Barker *et al.* (1961), or by paper electrophoresis (Davidson & Smellie, 1952). Solutions of nucleotides eluted from the ion-exchange column were evaporated under reduced pressure and finally freeze-dried. The purine nucleotides (approx. 5 mg.) were dissolved in 1 ml. of N-HCl and heated at 100° for 1 hr. Ribose was prepared from the pyrimidine nucleotides by the following adaptation of the method of Itzhaki (1960; and personal communication). Approximately 5 mg. of nucleotide was dissolved in 1 ml. of 0.1N-NaHCO₃. Bromine water, made by diluting freshly prepared saturated aq. bromine with 2 vol. of water, was added until a permanent colour of bromine remained (approx. 0.5-1 ml.). The solution was allowed to stand at room temperature for 20 min. and excess of bromine was removed by blowing a jet of N₂ on to the surface of the solution. The solution was brought to approx. 0.05N with respect to NaOH by addition of N-NaOH and was allowed to stand at room temperature for 1 hr. The solution was then made normal with respect to acid by addition of 10N-HCl and the resulting solution was heated at 100° for 1 hr.

The ribose-containing solutions from purine or pyrimidine nucleotides produced by the above methods were neutralized with aq. N-NH₃ soln., passed down a column of mixed resins containing approx. 10 g. of wet resin, and washed through with 10 ml. of water. The eluates were concentrated under reduced pressure to not more than 1 ml. and were chromatographed on Whatman no. 3 MM paper with the system ethyl acetate-acetic acid-water (3:1:3, by vol.) (Jermyn & Isherwood, 1949). Concentrations and radioactivities of ribose were determined on portions of eluates from the appropriate areas of the paper, located by spraying a longitudinal strip (cf. Jermyn & Isherwood, 1949). In some experiments, ribose was prepared from the pooled purine and pyrimidine nucleotides respectively as follows. Approx. 25 mg. of sodium nucleate was heated at 100° with 1 ml. of N-HCl for 1 hr. The solution was diluted to 15 ml. and passed successively through a column of Amberlite IR-45 and a column of mixed resin. The resulting solution, containing ribose derived from the purine nucleotides, was subjected to paper chromatography as described above. The pyrimidine nucleotides were eluted from the column of Amberlite IR-45 with aq. N-NH₃ soln. until no

further ultraviolet-absorbing material was obtained. The eluates were evaporated under reduced pressure and treated with bromine water as described above.

RESULTS

Utilization of [U-¹⁴C]glucose. A culture (500 ml.) of *C. utilis* was grown overnight and harvested by centrifugation at 3000g and 5°. A suspension was made of the cells in 600 ml. of fresh medium, which was divided equally between two flasks, to one of which was added 15 mg. of adenine dissolved in 1 ml. of water. Both flasks were aerated at 30° and samples were withdrawn at intervals for measurement of turbidity. When growth was proceeding logarithmically, 2.5 ml. of a solution of [U-¹⁴C]-glucose containing approx. 20 μ C/ml. was added to each flask and aeration was continued at 30° until the cell density had approximately doubled after addition of the radioactive material. The course of the growth was the same in the experimental and control flasks. The cells from the two flasks were separately harvested as above, washed by centrifugation with 0.9% (w/v) sodium chloride, and the sodium nucleates were extracted as described in the Materials and Methods section. The time between cessation of aeration and the beginning of the extraction (approx. 35 min.) was the same for experimental and control cultures. Radioactivity was measured in samples of the sodium nucleates. In Expts. 1 and 2 (strain IMI 23311), and in Expt. 3 (strain NCYC 321), the specific activities, expressed as counts/min./mg., of the sodium nucleates from cells grown in normal medium were respectively $544 \pm 1.5\%$, $538 \pm 1.5\%$ and $734 \pm 1\%$; the specific activities of sodium nucleates from corresponding cultures grown in the adenine-enriched medium were respectively $432 \pm 1.5\%$ (20.6), $445 \pm 1.5\%$ (17.4) and $552 \pm 1\%$ (24.8%). Each figure is the average of three determinations. The variation between planchets was as indicated. The figures in parentheses are the percentage reductions in specific activity produced by the adenine.

Samples of sodium nucleates were hydrolysed with potassium hydroxide, the nucleotides separated as described in the Materials and Methods section and the specific activities of the mixed nucleoside 2'- and 3'-phosphates were measured. In Expts. 2 and 4 ribose was prepared from each nucleotide fraction and the specific activities were measured as described in the Materials and Methods section. The results are shown in Table 1.

Utilization of [1-¹⁴C]glucose and [6-¹⁴C]glucose. *C. utilis* was grown in the presence of the labelled glucose and sodium nucleates were prepared as described above, with the exception that cultures were aerated with carbon dioxide-free air (see below). Purine-bound ribose and pyrimidine-

bound ribose were prepared as described in the Materials and Methods section. The specific activity of each sample of ribose is shown in Table 2.

In each culture containing [1^{14}C]glucose or [6^{14}C]glucose the carbon dioxide evolved was absorbed in a series of three wash-bottles containing filtered aqueous $\text{Ba}(\text{OH})_2$ soln. No barium carbonate was formed in the third bottle. At the end of the experiment, the first two bottles in each train were sealed, transferred to a carbon dioxide-free chamber, the barium carbonate was collected by filtration and the specific activity determined. The results are shown in Table 2.

Respiration of C. utilis and assimilation of glucose.
Uptake of oxygen by cultures of *C. utilis* containing

equal numbers of cells was measured in normal and glucose-free media in the presence and in the absence of a supplement of adenine. The adenine affected neither the endogenous respiration nor respiration in the presence of glucose.

Cultures of *C. utilis* containing equal numbers of cells were grown in aerated medium with and without the addition of adenine. Samples were withdrawn at intervals and, after removal of cells and deionization by passage through a column of mixed-bed resin, glucose was determined in portions of the medium. No difference was observed over a period of 4 hr. in the assimilation of glucose from the medium by cells grown in normal and adenine-enriched medium.

Table 1. *Effect of adenine on the incorporation of ^{14}C into nucleotides and ribose of RNA in Candida utilis growing in the presence of [$U^{14}\text{C}$]glucose*

Sodium nucleates, extracted from exponentially growing cultures of *C. utilis*, were hydrolysed with alkali and the nucleotides were separated by ion-exchange chromatography. Ribose was prepared from nucleotide fractions as described in the Materials and Methods section. Specific radioactivities of samples of both nucleotides and ribose were measured. Figures in parentheses are the percentage reduction of activity produced by the adenine.

	Sp. activity (counts/min./mg.)							
	Adenine enriched				Control			
	Expt. 1*	Expt. 2*	Expt. 3†	Expt. 4*	Expt. 1*	Expt. 2*	Expt. 3†	Expt. 4*
Adenine nucleotides	5740 (48)	10020 (14)	738 (26)	—	10980	11760	1003	—
Adenine-bound ribose	—	524 (16)	—	283 (57)	—	625	—	655
Guanine nucleotides	2980 (57)	9500 (16)	516 (43)	—	6995	11300	913	—
Guanine-bound ribose	—	537 (17)	—	284 (59)	—	650	—	700
Cytosine nucleotides	12210 (15)	7500 (52)	1234 (-21)	—	14320	15560	1022	—
Cytosine-bound ribose	—	406 (42)	—	190 (64)	—	704	—	522
Uracil nucleotides	6110	15480 (-7)	941 (-12)	—	Lost	15050	845	—
Uracil-bound ribose	—	635 (10)	—	352 (56)	—	708	—	798
* Strain IMI 23311.				† Strain NCYC 321.				

Table 2. *Effect of adenine on the utilization of [1^{14}C]glucose and [6^{14}C]glucose by Candida utilis*

Sodium nucleates, prepared from cells of strain IMI 23311 growing exponentially in the presence of the radioactive glucose and aerated with CO_2 -free air, were hydrolysed and ribose was prepared separately from the combined purine nucleotides and combined pyrimidine nucleotides as described in the Materials and Methods section. Respired CO_2 was collected as BaCO_3 . Figures in parentheses are the percentage reduction in specific activity produced by the adenine.

Material analysed	Sp. activity of ribose (counts/min./mg.) or BaCO_3 ($\mu\text{C/g.}$)			
	Expt. 5 ([1^{14}C]glucose)		Expt. 6 ([6^{14}C]glucose)	
	Adenine enriched	Control	Adenine enriched	Control
	Purine-bound ribose 793 (16)	840	2560 (14)	2960
Pyrimidine-bound ribose	457 (58)	1090	96 (33)	124
BaCO_3	5.11	5.05	1.77	1.48

Quantity and composition of RNA formed. Sodium nucleates were prepared from suspensions of exponentially growing cultures of *C. utilis*, the numbers of cells in which were determined after dilution of a sample. Total phosphorus was determined in a fraction of the sodium nucleates. The RNA phosphorus in cells grown in normal medium was 52.5 ± 1.3 mg./ 10^{12} cells; those grown under identical conditions but with the addition of adenine contained 51.5 ± 1.0 mg./ 10^{12} cells.

The adenine, guanine, cytosine and uracil nucleotides obtained from sodium nucleates derived from cells grown in normal and enriched medium were separated by paper electrophoresis. The four nucleotide fractions were eluted from appropriate areas of the paper and the molar proportion of each was determined spectroscopically. The molar proportions of adenine, guanine, cytosine and uracil nucleotides in RNA from cells grown in normal medium were 16.9:36.6:32.6:13.9 respectively; in RNA from cells grown in enriched medium, the corresponding proportions were 14.9:38.7:33.7:12.7.

DISCUSSION

In all six experiments it was found that enrichment of the growth medium with adenine resulted in a reduction in the labelling of the RNA by radioactive glucose. In the three experiments in which the specific activity of the whole RNA was measured, the reduction was found to be about 20%. Although reduction in the labelling of the whole RNA is consistently observed, the results expressed in Tables 1 and 2 show considerable variation in the effect on the labelling of the individual nucleotides. It was not feasible to determine the errors associated with the analytical procedures in each experiment but, in Expt. 3, determinations of the specific activities of nucleotides prepared from the same sample of RNA showed variations for the cytosine, adenine, guanine and uracil nucleotides of 1, 2.5, 4 and 4% respectively. Furthermore, two samples of RNA from the same yeast culture had specific activities differing by 5%. Thus variations in the effects produced by adenine are not due to experimental errors. Differences between experiments are believed to be due to difficulties in controlling the conditions of incorporation. Although corresponding experimental and control cultures showed the same rate of growth and were subjected to the same conditions of incorporation, these factors varied from one experiment to another. Incorporation was allowed to take place under conditions of rapid growth, and small changes in the rate of growth and duration of incorporation might be expected to result in relatively large variations in the labelling obtained. Differences in the degree of

labelling between control experiments are believed to be due to this. For reasons discussed below and in the next paper, it is believed that the effect of adenine on the utilization of glucose is due to at least two, and probably more, kinetically independent causes. Thus, under conditions of rapid growth and incorporation, variations in the magnitude or even direction of the effect might well be expected, particularly if the individual nucleotides exhibit different rates of turnover.

It is seen that in Expt. 2 the reduction in the labelling of the whole nucleotides corresponds to that in the ribose derived from them, and this strongly suggests that the effect is associated largely, if not entirely, with the ribosyl residues of the nucleotides. The effect does not appear to be due to a general toxicity of adenine since no alteration of the rates of growth and respiration was observed, and no morphological changes were discernible. Further, there seems to be no general suppression of RNA synthesis, since not only is the rate of uptake of glucose from the medium unchanged, but the quantity and composition of the RNA obtained is unaltered.

Reduction in the extent of labelling of ribose by radioactive glucose could arise either from changes in the rate of turnover, which would be difficult or impossible to assess (cf. Mandelstam, 1960), or by dilution of the label by either exogenous or endogenous carbon. The only exogenous sources other than glucose are adenine and carbon dioxide. No estimate has been made of the extent of carbon dioxide fixation, but it seems unlikely that it would be sufficient to account for the observed results. Furthermore, it is known that exogenous adenine is effectively incorporated into the adenine and guanine residues of RNA in *C. utilis* (Barker *et al.* 1961). Thus, by virtue of nucleotide material formed before the addition of labelled glucose, adenine may reduce the subsequent requirements of the cell for glucose or its metabolites. The report that adenine stimulates nucleotide formation in Ehrlich ascites cells (Ellis & Scholefield, 1962) is in accord with this interpretation. The nature and the rate of the initial reactions concerned in the assimilation of adenine are under investigation.

Dilution of the label by endogenous carbon could arise by mobilization of carbon reserves in the cell, but adenine does not affect the endogenous respiration. Alternatively, the reduction in labelling may be due to an alteration of the contributions of various metabolic pathways concerned in the synthesis of ribose in such a way that larger pools of intermediates are involved. The reactions concerned in the formation of ribose from glucose in *C. utilis* are discussed in the next paper. However, in view of the results with $[1-^{14}\text{C}]$ glucose and $[6-^{14}\text{C}]$ glucose recorded in this paper, the following

observations may be made. Since the experiments were carried out under identical conditions with the same amounts of radioactive glucose, the specific activities of barium carbonate shown in Table 2 are proportional to the relative specific activities. Thus the higher specific activity of the carbon dioxide released in the presence of [1-¹⁴C]-glucose than that in the presence of [6-¹⁴C]glucose (Table 2) is in accord with the predominance of oxidative decarboxylation of glucose 6-phosphate. The low specific activity of pyrimidine-bound ribose formed from [6-¹⁴C]glucose compared with purine-bound ribose formed in the same cultures may be due either to a higher turnover rate in the purine nucleotides or to the existence of more than one precursor pool of nucleotide ribose. This point is discussed further in the next paper.

The conclusion is reached that enrichment of the exogenous adenine results in a significant depression in the labelling of whole cellular RNA by radioactive glucose in *C. utilis*. This is largely, if not entirely, connected with the synthesis of ribose. The variations in the results observed in the hydrolysis products of RNA suggest that the effects of adenine are due to disturbances in a number of kinetically independent metabolic processes, the relative importance of which vary with differences in experimental conditions. Further work is required to establish their nature.

SUMMARY

1. Addition of adenine to the culture medium causes a reduction in the specific activity of the RNA of *Candida utilis* growing exponentially with [¹⁴C]glucose as sole carbon source.

2. Alterations in the specific activities of the nucleotides and ribose derived from the RNA show variations between experiments.

3. The percentage reductions in specific activities of the nucleotides and the ribose derived from them are similar, suggesting that the effect of adenine is largely concerned with the formation of ribose.

4. The specific activity of respired carbon dioxide is not affected by the addition of adenine. The specific activity of carbon dioxide from yeast growing in presence of [1-¹⁴C]glucose is approximately three times that from yeast growing in presence of [6-¹⁴C]glucose, suggesting the predominance of oxidative decarboxylation of glucose 6-phosphate.

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Biosynthesis of Polynucleotides

6. THE UTILIZATION OF GLUCOSE IN THE SYNTHESIS OF RIBOSE IN *CANDIDA UTILIS*

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Addition of adenine to the culture medium results in a depression of the labelling of RNA in *Candida utilis* growing exponentially in the presence of radioactive glucose as the sole carbon source (Barker, Jechová, Nicholson & Thompson, 1964).

Evidence suggests that the effect may be due in part to changes in the contribution of the various metabolic pathways concerned in ribose formation. The reactions involved in the formation of ribose in *C. utilis* have not been fully defined. Sowden,