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*

By G. R. BARKER AND B. H. NICHOLSON

Department of Chemistry, The University, Manchester 13

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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,

Frankel, Moore & McClary (1954) found very low activity in the ribose derived from the RNA of *Torula utilis* supplied with [$1\text{-}^{14}\text{C}$]glucose. (The names *Torula utilis* and *Candida utilis* are taken as synonymous; cf. Skinner & Fletcher, 1960.) David & Renaut (1954), using the same organism, found that 93% of the activity in ribose derived from [$2\text{-}^{14}\text{C}$]glucose was present in the first three carbon atoms of the chain. In a subsequent experiment, David & Renaut (1955) found that all the activity was located at C_{11} of the ribose molecule. Thus there is some evidence that the oxidative pathway from glucose to ribose plays a major role in this organism, and this is in agreement with results presented by Barker *et al.* (1964). In the present studies, an attempt has been made to investigate the part played by major and minor contributory processes, and their possible susceptibility to exogenous adenine.

METHODS AND MATERIALS

Growth of organisms. *Candida utilis*, strain IMI 23311, obtained from the Commonwealth Mycological Institute, was maintained on agar slopes and grown in liquid medium as described by Barker, Hignett, Jackson & Wadsworth (1961). The time required to give a twofold increase in extinction at $670\text{ m}\mu$ was approx. 5 hr. Cells were harvested by centrifuging at $3000g$ and 5° .

Lactobacillus plantarum strain 8026 (National Collection of Industrial Bacteria) was maintained by transfers of stab cultures, and suspensions of resting cells were obtained in a xylose-containing medium as described by Gest & Lampen (1952).

Purification of nucleotides and ribose from RNA of C. utilis. Methods for the extraction of sodium nucleates, hydrolysis, separation of nucleotides and the preparation of ribose from them were as described by Barker *et al.* (1964). To avoid contamination of one nucleotide by another, the graph of extinction at $260\text{ m}\mu$ against volume of eluate from the ion-exchange column was extrapolated to zero extinction for each peak and only those fractions were taken which showed no overlap between peaks. Ribose was purified by chromatography on sheets of Whatman no. 3 MM paper in the solvent system ethyl acetate–water–acetic acid (3:3:1, by vol.) (Jermyn & Isherwood, 1949), up to 10 mg. of material being loaded on to paper 22 cm. wide.

Degradation of ribose. The sample of ribose dissolved in 1 ml. of water was added to 2 ml. of a suspension of *L. plantarum*, containing approx. 0.5 g. of wet cells, and 40 ml. of 42 mM- NaHCO_3 in a 60 ml. Warburg flask. The flask was connected to a manometer, gassed for 15 min. with $\text{N}_2 + \text{CO}_2$ (95:5, v/v), and then shaken at 100 strokes/min. at 37° . When evolution of CO_2 reached a constant low level (after approximately 4.5 hr.), 2 ml. of 2N- H_2SO_4 was added and the cells were removed by centrifuging. Under these conditions, approx. 0.5 m-mole of ribose was fermented in 4.5 hr.

The supernatant from the cells was steam-distilled as described by Gest & Lampen (1952). The distillate was collected in 10 ml. fractions, which were separately titrated

with N-NaOH to the end point of bromothymol blue. A graph of log ($\mu\text{l. of NaOH}$) used in each titration against total volume of distillate gave a plot of variable slope, indicating the presence of more than one acidic species. The neutralized distillates were combined, concentrated to 20 ml. (cf. Gest & Lampen, 1952), brought to pH 2 by addition of 2N- H_2SO_4 and again steam-distilled. Titration of 10 ml. fractions and graphical examination of the results as above gave a plot having a constant negative slope, indicating the homogeneity of the distillate. Acetic acid was isolated from the second distillate by continuous extraction with ether for 17 hr. and removal of the solvent, giving an almost quantitative recovery. Lactic acid was similarly recovered from the combined residues of the two steam-distillations. Determination of lactic acid in the ether extracts by the method of Barker & Summerson (1941) indicated a recovery of 96% from the fermentation. Final purification of the two acids was effected by chromatography on Amberlite CG-50 ion-exchange resin (Rohm and Haas Co., Philadelphia, U.S.A.) following the method of Seki (1958). In trial experiments it was found that complete separation of the acids from one another could be achieved under the conditions used.

Degradation of lactic acid and acetic acid. Lactic acid was oxidized with chromic acid (Wood, Lifson & Lorber, 1945) to yield acetic acid and CO_2 , which was recovered as BaCO_3 . Acetic acid, either from the fermentation of ribose or from the decarboxylation of lactic acid, was degraded by the Schmidt reaction (Anker, 1957). Carbon dioxide was collected as BaCO_3 , the reaction mixture was made alkaline by the addition of 30% (w/v) NaOH and methylamine was distilled and absorbed in 2 ml. of N-HCl. The methylamine was converted into 2,4-dinitromethylaniline, which was purified by chromatography (Birch, Moye, Rickards & Vanek, 1962).

Measurement of radioactivity. Specific radioactivities were measured with a thin end-window Geiger-Müller counter (thickness of window 1.7 mg./cm. 2). Infinitely thick samples (not less than 20 mg./cm. 2) of either BaCO_3 or 2,4-dinitromethylaniline were counted until the statistical error was not greater than 5% and compared with a standard planchet of known specific activity. All specific activities of 2,4-dinitromethylaniline were multiplied by the experimentally determined factor of 1.06 to make them comparable with those of BaCO_3 .

RESULTS

C. utilis was grown in 500 ml. of liquid medium, harvested and the cells were suspended in 600 ml. of fresh medium as described by Barker *et al.* (1964). The cell suspension was divided equally between two flasks; 15 mg. of adenine dissolved in 1 ml. of water was added to one flask and the two cultures were aerated at 37° . When exponential growth had begun, 2.5 ml. of a solution of [$2\text{-}^{14}\text{C}$]glucose containing approx. 100 $\mu\text{C}/\text{ml.}$ (The Radiochemical Centre, Amersham, Bucks.) was added to each flask and aeration was continued at 37° for 5.5 hr. The cells were harvested, sodium nucleates were extracted and hydrolysed and the mixed nucleoside 2'- and 3'-phosphates were separated by

Table 1. *Incorporation of isotope from [2-¹⁴C]glucose into ribose of RNA in Candida utilis*

C. utilis was grown in the presence of [2-¹⁴C]glucose. RNA was isolated and ribose prepared from the constituent nucleotides was degraded as described in the text. The specific activities of BaCO₃ from C₍₂₎, C₍₃₎ and C₍₄₎ and of 2,4-dinitromethylaniline from C₍₁₎ and C₍₅₎ were measured. Sp. activities in parentheses are given as counts/min./mg.

Origin of ribose	Medium	Sp. activity (rel. to that from C ₍₁₎ taken as 100)				
		C ₍₁₎	C ₍₂₎	C ₍₃₎	C ₍₄₎	C ₍₅₎
Cytidylic acids	Normal	100 (120)	11.3	2.1	11.8	7.2
	Enriched	100 (108)	10.5	3.1	12.8	2.9
Adenylic acids	Normal	100 (158)	13.0	3.5	15.4	8.8
	Enriched	100 (98)	11.3	3.2	14.1	2.6
Guanylic acids	Normal	100 (195)	13.6	4.0	15.8	8.1
	Enriched	100 (94)	12.0	3.1	17.2	2.2
Uridylic acids	Normal	100 (179)	7.8	2.7	10.7	5.2
	Enriched	100 (95)	11.2	3.1	13.2	7.1

ion-exchange chromatography. Each nucleotide fraction was diluted approximately threefold with carrier nucleotide, and ribose was prepared from it. To the solution of ribose obtained from each nucleotide was added approx. 0.5 m-mole of carrier ribose and the material was fermented with *L. plantarum*. After separation of acetic acid and lactic acid, they were separately degraded to give barium carbonate and 2,4-dinitromethylaniline, as described in the Materials and Methods section. The specific activities were measured and are shown in Table 1. All activities are shown relative to the carbon from C₍₁₎ taken as 100 to indicate the distribution of isotopic carbon in the ribose molecule. The observed specific activities of carbon derived from C₍₁₎ of ribose are given in parentheses to indicate the levels of activity measured. Since carrier materials were measured only approximately, differences between figures in parentheses for experimental and control material are not significant.

DISCUSSION

The most direct route for the synthesis of pentose from hexose is the oxidative pathway whereby glucose 6-phosphate is oxidized and decarboxylated to give ribulose 5-phosphate. A second route involves the reactions catalysed by transaldolase and transketolase and may be summarized:

These two series of reactions are generally regarded as the most significant in the production of ribose and various attempts have been made to assess their respective contributions in this process.

The distribution of isotopic carbon in ribose derived from labelled glucose can be predicted with certainty if reactions of the transaldolase-transketolase network are absent. If, however, such reactions operate either for the synthesis of pentose as in reactions (1), (2) and (3) above, or for the dissimulation of hexose (cf. Dawes & Holms, 1958; Katz & Wood, 1960; Wood, 1955), some pentose is reconverted into hexose in which the distribution of isotope is different from that in the original labelled molecule. The distribution of label in ribose may be compared with that in, for instance, glycogen, which may be assumed to reflect the true distribution in the glucose acting as the precursor of ribose. The validity of this method depends on the assumption that equilibration between the labelled glucose and glycogen is more rapid than the processes involved in ribose synthesis, which may not be true (Sowden *et al.* 1954). Szykiewicz, Sable & Pflueger (1961) partly avoided the problem by confining their measurements of activity to C₍₁₎ and C₍₂₎ of ribose and C₍₁₎, C₍₂₎ and C₍₃₎ of glucose. The first two carbon atoms of ribose are labelled only by the first three carbon atoms of glucose, and no account need therefore be taken of the origin of the glyceraldehyde phosphate. This method is not appropriate for the present purpose, which is to explain the overall depression of the labelling of ribose.

Glyceraldehyde phosphate is required in stoichiometric amounts for the complete operation of reactions (1) and (2)–(3) above. Pontremoli, Bonsignore, Grazi & Horecker (1960) have demonstrated the existence of a concerted attack by transaldolase and transketolase on fructose 6-phosphate in which no glyceraldehyde phosphate is required. The products are pentose 5-phosphate and heptulose

7-phosphate and the overall reaction is equivalent to the sum of reactions (1) and (2) above:

The steady-state production of ribose by this reaction is also likely to require stoichiometric quantities of glyceraldehyde phosphate to remove the sedoheptulose 7-phosphate. Thus the participation of glyceraldehyde phosphate must be taken into consideration and the production of pentose from hexose must be viewed in relation to the metabolism of glucose as a whole.

Glyceraldehyde phosphate produced by the combined operation of the oxidative pathway and the transaldolase-transketolase network is derived solely from $C_{(4)}$, $C_{(5)}$ and $C_{(6)}$ of glucose. On the other hand, if glucose is also metabolized by the Embden-Meyerhof pathway, glyceraldehyde phosphate will also arise from $C_{(1)}$, $C_{(2)}$ and $C_{(3)}$ of glucose, $C_{(1)}$ of glyceraldehyde phosphate corresponding to $C_{(3)}$ of glucose, provided that equilibrium is set up between the two triose phosphates.

Metabolism by the Entner-Doudoroff pathway if it occurs in this organism would result in glyceraldehyde phosphate derived from the same carbon atoms of glucose, but in which $C_{(1)}$ of glyceraldehyde phosphate corresponds to $C_{(1)}$ of glucose.

As a result of these considerations it can be seen that ribose can arise from glucose having the distributions of carbon atoms indicated in Scheme 1. According to this Scheme, label from $[2-^{14}\text{C}]$ glucose is expected at $C_{(1)}$ of ribose according to reactions (a), (b) and (c), at $C_{(2)}$ by reactions (d) and (e) and at $C_{(1)}$ and $C_{(2)}$ by reactions (f). Operation of the Embden-Meyerhof or Entner-Doudoroff pathway in conjunction with the transaldolase-transketolase network would result in label from $[2-^{14}\text{C}]$ glucose appearing at $C_{(4)}$ of ribose if equilibrium is set up between the two triose phosphates. None of these pathways gives rise to activity at $C_{(3)}$ or $C_{(5)}$ of ribose.

On the basis of Scheme 1, the results expressed in Table 1 lead to the conclusion, in agreement with Sowden *et al.* (1954) and David & Renaut (1954, 1955), that the principal route from glucose

$\begin{bmatrix} \text{C} & \text{C} & \text{C} \\ 4 & 5 & 6 \end{bmatrix}$ may be replaced by $\begin{bmatrix} \text{C} & \text{C} & \text{C} \\ 1 & 2 & 3 \end{bmatrix}$ or $\begin{bmatrix} \text{C} & \text{C} & \text{C} \\ 3 & 2 & 1 \end{bmatrix}$ if the Entner-Doudoroff or Embden-Meyerhof pathway is involved.

Scheme 1. Derivation of carbon atoms of pentose 5-phosphate formed from glucose 6-phosphate by oxidative decarboxylation ($\longrightarrow$) and by combined actions of transaldolase and transketolase ($\longrightarrow$).

to ribose is the oxidative pathway. The label at $C_{(2)}$ is explained by the participation of the transaldolase-transketolase network, and that at $C_{(4)}$ suggests that this network operates in the production of ribose, at least in part, by utilization of glyceraldehyde phosphate arising from the Embden-Meyerhof or Entner-Doudoroff pathway. In agreement with the Scheme discussed above,

Similarly:

Scheme 2. Effect of the reversal of the aldolase reaction on the distribution of carbon atoms of glucose 6-phosphate in pentose 5-phosphate formed by oxidative decarboxylation and combined actions of transaldolase and transketolase.

activity at $C_{(3)}$ is low, but the fact that activity at $C_{(5)}$ is two or three times that at $C_{(3)}$ is not explicable on the basis of the above considerations. Labelling at $C_{(5)}$ may be due to redistribution of isotope in the hexose re-formed from ribose, hexose 6-phosphate (A) and hexose 6-phosphate (B) (Scheme 1), caused by the reversal of the aldolase reaction, in conjunction with equilibration of the triose phosphates. In this case, $C_{(1)}$ and $C_{(6)}$ of any of the hexose monophosphate molecules in Scheme 1 may be assumed to be interchanged, label originally at $C_{(1)}$ of the hexose thus being located at $C_{(6)}$ of ribose after oxidative decarboxylation (Scheme 2). An alternative explanation of the activity at $C_{(5)}$ might be found in the reversal of the chain of a C_5 unit, but as far as we are aware a complete series of reactions by which this could be achieved is not known. It is therefore preferred to assume that hexose phosphate is re-formed from triose phosphate and, as can be seen from Scheme 2, this also provides a mechanism for the limited introduction of isotope at $C_{(3)}$. The operation of these reactions on the original glucose 6-phosphate would also contribute label at $C_{(4)}$ of ribose.

The results expressed in Table 1 show no gross differences between the distributions of label in the ribose derived from the four nucleotides of RNA in cells growing in normal medium. This indicates that the ribosyl residues of all the nucleotides are derived from one pentose phosphate pool. It is therefore concluded that the differences previously observed between the specific activities of ribose obtained from the four nucleotides (Barker *et al.* 1964) are due to variations in the rate of turnover.

From Table 1 it is seen that the pattern of labelling of the ribosyl residues of RNA derived from cells grown in presence of adenine is remarkably similar to that for cells grown in normal medium. The main difference concerns the activity at $C_{(5)}$ of the ribose from adenylic acid, guanylic acid and cytidylic acid. In view of the above discussion of the mechanism of the labelling of $C_{(5)}$ of ribose by isotope originally located at $C_{(2)}$ of glucose, it is tentatively suggested that the effect of adenine on the carbohydrate metabolism of *C. utilis* may be, in part, associated with the aldolase reaction and its reversal. However, if the effect of adenine were due only to the elimination of the re-formation of hexose from C_3 units, a reduction of the activity at $C_{(3)}$ of ribose might also be expected. Thus this does not provide a full explanation of the results obtained.

SUMMARY

1. *Candida utilis* has been grown in the presence of $[2-^{14}C]$ glucose, and ribose has been prepared from the cytosine, adenine, guanine and uracil nucleotides obtained from the RNA.

2. Ribose from each nucleotide has been degraded and the distribution of isotope has been determined.

3. The distribution of isotope suggests that oxidation and decarboxylation of glucose 6-phosphate is largely responsible for synthesis of ribose.

4. Other reactions that contribute to ribose synthesis probably include those catalysed by transaldolase, transketolase, aldolase and triose phosphate isomerase.

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The Time Course of the Recombination of Human Adult and Canine Haemoglobins

BY E. R. HUEHNS AND E. M. SHOOTER

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

AND G. H. BEAVEN

Medical Research Council Laboratories, Hampstead, London, N.W. 3

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The composition of the new haemoglobin species formed in a wide variety of recombination experiments by using either acid or alkaline conditions to bring about dissociation (Itano, Singer & Robinson, 1959; Vinograd, Hutchinson & Schroeder, 1959; Gammack, Huehns, Lehmann & Shooter, 1961) suggests that they have arisen by a simple exchange of α_2 - and β_2 -sub-units between the two parent haemoglobins as indicated by the equation:

Moreover, the formation, in the recombination of carbonmonoxyhaemoglobin S and methaemoglobin C (Singer & Itano, 1959) of species which behave electrophoretically as though both α -chain haem groups are in the carbonmonoxy-form and both β -chain haem groups in the met-form, and vice versa, but not of species where one α -chain (or one β -chain) carried carbonmonoxy-haem and the

other the ferrihaem group, appears to provide direct evidence in favour of this mechanism. There are, however, a number of experiments that are difficult to explain on this basis; these have been summarized by Antonini, Wyman, Bucci, Fronticelli & Rossi-Fanelli (1962).

One of them is the finding that, although the initial dissociation of each haemoglobin into half-molecules occurs rapidly, it is necessary with certain mixtures of haemoglobins, e.g. normal human adult and canine haemoglobins (Robinson & Itano, 1960) and abnormal human haemoglobins and haemoglobin A₂ (Huehns & Shooter, 1961), to keep them together at either acid or alkaline pH for a long time before maximum exchange of sub-units occurs. It could be argued that the rate of exchange is here dependent on the occurrence of a slow conformational change in one or both of the α_2 - and β_2 -sub-units of normal adult or canine