Metabolic Functions of Myo-inositol

II. EFFECT OF INOSITOL DEFICIENCY ON THE METABOLISM OF NUCLEIC ACIDS OF MAMMALIAN CELLS IN TISSUE CULTURE*

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We have previously reported (1) that myo-inositol deficiency in yeast results in a marked alteration of the chemical composition of the cell wall, as a consequence of which cell division is inhibited. The main change observed in these studies involved the polysaccharide fraction of the cell walls and it was inferred that inositol deficiency interferes with the normal biosynthesis of cell wall polysaccharides. Since inositol is required for the maintenance and multiplication of mammalian cells grown in vitro (2), and since the cell walls of mammalian cells do not contain the polysaccharides found in yeast cell walls, it would appear that inositol may exhibit different functions in mammalian cells from those observed in yeast.

The present report deals with the effects of inositol deficiency on the metabolism of nucleic acids of mammalian cells in tissue culture. It has been found that inositol deficiency causes a marked decrease of the acid-soluble nucleotides and the ribonucleic acid of the cells, as well as a significant decrease of the rate of incorporation of radioactive carbon of glucose into the guanine of ribonucleic acid. The amount and the specific activity of the acid-soluble nucleotides, the ribonucleic acid bases, and the deoxyribonucleic acid bases have been determined at various periods with cells grown with and without inositol.

EXPERIMENTAL PROCEDURE

Growth Medium and Culture Conditions—The cells used in the present studies were the progeny of an inositol-requiring KB strain originally obtained from Dr. H. Eagle of the National Institutes of Health. It has been maintained in our laboratory for 18 months as monolayer cultures adherent to glass and as suspension cultures. In all of the experiments described in the present paper, the cells were grown as suspension cultures in spinner flasks with the use of Eagle’s medium (3) with the following modifications. The concentration of glucose, glutamine, and vitamins was twice that used by Eagle. The medium was supplemented with 10% dialyzed horse serum and was replaced every 3 days. The cell density was maintained between \(1 \times 10^4\) and \(3 \times 10^5\) cells per ml. The deficient medium was of a similar composition but without added inositol.

Materials—Horse serum was obtained from Cappel Laboratories, Inc., and was dialyzed for 24 hours against 20 volumes of a 0.68% solution of NaCl at 4°C. During this time the saline solution was changed four times. Glucose-C\(^4\) uniformly labeled, was obtained from Volk Radio-Chemical Company. Its specific activity was \(3.7 \times 10^6\) c.p.m. per mg of glucose. Enough radioactive glucose was added to the culture medium so that the resulting specific activity of glucose was \(9 \times 10^6\) c.p.m. per \(\mu\)mole. l-Amino acids were obtained from Mann Research Laboratories, Inc., and the vitamins from Nutritional Biochemicals Corporation. All other materials were commercially available products of reagent grade. The spinner flasks were purchased from Boleco Glass, Inc.

Cell Morphology and Vital Staining—Cells were examined by phase microscopy for gross cytological alterations, with a Spencer model X2TV-HBW microscope. Cell counts were performed with a Levy counting chamber. Trypan blue was used as a vital stain to detect damaged and nonviable cells (4) which stain blue in contrast to normal cells which remain colorless.

Sterility Tests—All cultures were routinely tested for bacterial contamination by subculturing in fluid thioglycolate medium obtained from Difco Laboratories. The absence of pneumo-pneumonia-like organisms from our cultures was confirmed by monthly tests.

Procedure for Estimation of RNA, DNA, and Protein—For the separation of the RNA, DNA, and protein of the cells, the method of Ogur et al. (5) was used. The amount of RNA was estimated by the orcinol method for pentose (6) and the results expressed as “RNA pentose” with pure adenosine 5'-phosphate as standard. DNA was estimated by Burton’s diphenylamine method (7) with, as reference standard, the Na salt of herring sperm DNA of 84% purity (based on phosphorus content of 7.7%). The values were corrected to 100% purity.

The residue remaining after removal of the nucleic acids contained the protein and this was estimated spectrophotometrically (1) with crystalline bovine albumin as reference standard.

Isolation and Estimation of Bases Derived from RNA, DNA, and Acid-Soluble Nucleotides—The cells were treated with cold trichloroacetic acid to give the trichloroacetic acid-soluble fraction containing the nucleotides and the trichloroacetic acid-insoluble material containing the nucleic acids and proteins (8). The nucleotides were adsorbed on a column of Dowex 1-X10 resin and, after elution with 0.1 N HCl (9), they were hydrolyzed to purine bases and pyrimidine nucleoside monophosphates (10). These were further separated by paper chromatography.

After removing the lipids from the trichloroacetic acid-insoluble material, the RNA was extracted by a method similar to that of Davidson and Smellie (11), except that HCl was used to neutralize the alkaline digest and trichloroacetic acid was...
added to a final concentration of 0.15 M. The resulting RNA nucleotides were separated from the precipitated DNA and protein by centrifugation and were isolated by the Norit method of Tsuboi and Price (12). They were then hydrolyzed to the free bases (13) and separated by paper chromatography. The DNA was extracted from the protein with hot trichloroacetic acid (5) and the resulting nucleotides were hydrolyzed to the free bases (13) and separated by paper chromatography.

Paper Chromatography and Spectrophotometry—Descending paper chromatography was carried out with isopropanol: HCl: H2O (65:16:19) as solvent. Standard reference compounds were chromatographed at the same time and the ultraviolet-absorbing spots were cut out and extracted with 0.1 N HCl for 16 hours at 35°. Recovery of the extracted compounds was better than 95%. The eluted substances were further identified by comparing their spectral properties with those of the eluted standard reference compounds (14).

Counting of Radioactive Samples—The samples were plated in stainless steel planchets at infinite thinness and were counted in a windowless gas counter. The over-all counting error was less than 5%.

RESULTS

Growth and Viability—When inositol was present in the medium, cell multiplication was maintained at an exponential rate with a doubling time of 35 hours and the viability, as measured by the vital staining method, was greater than 95%. In the absence of inositol, the rate of multiplication decreased constantly until it became zero between the fourth and the fifth day (Fig. 1). The viability of the cells began to drop from the first day of inositol deficiency and continued to drop steadily until it became 46% on the fifth day. In order to maintain the cells for longer periods of time, it was necessary to add, on the fifth day, 0.3 μg of inositol per ml of culture medium at a cell density of 2 x 10⁶ cells per ml. This addition of inositol can be repeated 1 week later without causing an increase of the cell population.

Cell Content of Acid-Soluble Nucleotides, RNA, DNA, and Protein—One of the most impressive effects of inositol deficiency is the decrease of the acid-soluble nucleotides and the RNA of the cells. The acid-soluble fraction of the KB cells contains nucleotides of adenine, guanine, and uracil. Of these, the adenine nucleotides comprise the highest proportion followed by the uracil nucleotides with a concentration 56% of that of the adenine nucleotides. The amount of guanine nucleotides is very small indeed, being 20% of the amount of the adenine nucleotides. It was not possible to detect any acid-soluble cytosine nucleotides even though the number of cells analyzed was 10 times greater than that used in the analyses for guanine nucleotides. Fig. 2 illustrates the early and progressive decrease of the acid-soluble nucleotides caused by inositol deficiency. It is seen that the concentration of all three nucleotides of the deficient cells dropped to 25% of that of the normal cells.

Concomitant with these changes of the acid-soluble nucleotides, there occurs a decrease of the RNA content of the inositol-deficient cells. This is illustrated in Fig. 3, together with data on the DNA content. It is shown that the DNA content of the cells was unaffected by inositol deficiency, but the RNA content decreased steadily to a level of about 36% of that of normal cells. The composition of the RNA was not affected by inositol deficiency as judged by the base ratios, which were as follows in both normal and deficient cells: adenine to guanine = 0.55 ± 0.01; cytosine to uracil = 2.72 ± 0.03; adenine + uracil to guanine + cytosine = 0.46 ± 0.01; adenine + guanine to uracil + cytosine = 1.38 ± 0.01. The decrease of cellular RNA affected equally the cytoplasmic, microsomal, and mitochondrial RNA.

The protein content of the cells was not affected by inositol deficiency and it varied between 9 and 11.5 μg per μg of DNA in both the normal and the deficient cells.

Incorporation of Uniformly Labeled Glucose-Cl4 into Proteins and Acid-Soluble Nucleotides—In view of the above changes in the amount of acid-soluble nucleotides and RNA of the cells brought about by inositol deficiency, it was decided to study this phenomenon in greater detail. Consequently, experiments were set up in which the rate of biosynthesis of cellular components was followed by the rate of incorporation of radioactivity from uniformly labeled glucose-C14. In these experiments, KB...
The ability of normal cells to incorporate glucose-carbon into proteins through known biochemical reactions is shown in Fig. 4. After an initial rapid rate, incorporation of C¹⁴ into cellular proteins proceeded at a slower but constant rate over a period of 10 days. In contrast to these findings, cells grown in the absence of inositol showed a significant decrease in the rate of labeling of their proteins to a level of 60% of the normal rate. This effect was manifest as early as the second day of inositol deficiency, and, after the sixth day, no further increase in the specific activity of the proteins could be observed. As mentioned previously, the protein content of the cells was not affected by the absence of inositol.

The incorporation of radioactive carbon from glucose into the acid-soluble nucleotides of the inositol-deficient cells proceeded at a rate similar to that of normal cells. This is shown in Fig. 5 in which the specific activities of UMP, guanine of guanine nucleotides, and adenine of adenine nucleotides are recorded over a period of 9 days. When the UMP was degraded to uracil, the specific activity of the isolated uracil was the same in both normal and deficient cells.

**DISCUSSION**

These studies demonstrate that one of the early effects of inositol deficiency is the decrease of the amount of acid-soluble

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Fig. 3. Amount of DNA and RNA pentose of KB cells grown with (O—O) and without (●—●) inositol.

Fig. 4. Specific activity of proteins of KB cells grown with (O—O) and without (●—●) inositol in the presence of uniformly labeled glucose-C¹⁴.

Fig. 5. Specific activities of the acid-soluble nucleotides of KB cells grown in the presence of uniformly labeled glucose-C¹⁴. Open characters are for cells grown with inositol whereas closed characters are for cells grown without inositol. O—O, UMP; △—△, guanine of guanine nucleotides; ■—■, adenine of adenine nucleotides.
nucleotides and the RNA of the cells. In the KB cells, as in the L cells (15), the adenine nucleotide pool is the main reservoir of purine compounds. It is 75% of the RNA adenine and 5 times as great as the pool guanine, which is 8.5% of the RNA guanine. In contrast to the L cells, the KB cells contain a significant pool of uracil nucleotides, the size of which is half of that of the adenine pool. As the cells become inositol-deficient, the size of pool nucleotides begins to decrease until it reaches a value one-fourth of the normal value on about the fourth day of inositol deficiency. Concomitant with these changes, there occurs a decrease of the cellular RNA to a level one-third of that of normal cells. Neither the DNA nor the protein content of the deficient cells shows any significant change from the normal values.

With uniformly labeled glucose-C\textsuperscript{14} it was possible to demonstrate that the rate of incorporation of C\textsuperscript{14} into the pool nucleotides was the same in both normal and deficient cells, even though the rate of cell multiplication of the deficient cells was considerably slowed down and was completely inhibited after 4 days' culture in the inositol-deficient medium. Similarly, the rate of incorporation of radioactivity into the RNA bases of the deficient cells proceeded at a normal rate in the cases of RNA adenine, cytosine, and uracil. In contrast to these findings, the RNA guanine was labeled at a rate half of that obtained with normal cells. The rate of labeling of the DNA bases of the deficient cells was uniformly decreased and it reflected the decreased rate of cell division.

The results discussed above indicate that there is a rapid turnover of RNA in the inositol-deficient cells, the rate of which is approximately one-third of the rate of synthesis of RNA in normal cells dividing at an exponential rate. This calculation was based on the following two observations: (a) the specific activities of the pool nucleotides and the RNA bases (with the exception of RNA guanine) were at all times of the same magnitude in both normal and deficient cells; and (b) when cell division was arrested as a result of inositol deficiency, the cellular RNA decreased to a level one-third of that of normal cells.

That this RNA turnover is not the result of cell turnover (cell multiplication followed by cell destruction) is evident from the fact that the specific activities of the DNA bases and of cellular proteins of the deficient cells were only a small fraction of those of the normal cells. Indeed, during the last 4 days of inositol deficiency, the specific activity of the cellular proteins and the DNA bases remained almost constant. Furthermore, the extracellular culture fluid did not contain any nucleic acids or nucleic acid precursors.

The observation that the specific activity of RNA guanine of the deficient cells was significantly lower than that of normal cells, even though the specific activity of pool guanine was the same in both normal and deficient cells, suggests that only a small fraction of the guanine pool serves as "precursor pool" for RNA guanine. Thus the specific activity of this "precursor pool guanine" must be significantly lower than that of the entire pool guanine, with the result that less C\textsuperscript{14} will be incorporated into RNA guanine. This interpretation is further strengthened by the observation that the specific activity of the entire pool guanine is not decreased by dilution with nonradioactive RNA guanine, since RNA guanine would equilibrate only with the very small "precursor pool guanine." Similar conclusions were reached by McFall and Magasanik (15) in their studies of the incorporation of adenine-8-C\textsuperscript{14} and guanosine-8-C\textsuperscript{14} into pool guanine and nucleic acid guanine of L cells. These authors concluded that the guanine pool is, for the most part, not involved in active metabolism.

The ability of L cells (15) and Hela cells (16) to interconvert adenine and guanine has also been reported.

Any mechanism by which the effects of inositol deficiency could be explained must take into account the three biochemical aberrations of nucleic acid metabolism described in the present report, namely, a decrease of the acid-soluble nucleotides, a decrease of the amount of cellular RNA, and a decrease in the rate of incorporation of C\textsuperscript{14} from glucose-C\textsuperscript{14} into RNA guanine. In view of the discussion presented above, we would like to propose as a possible mechanism that inositol deficiency causes a decrease of the rate of biosynthesis de novo of acid-soluble nucleotides, and, in particular, of pool guanine nucleotides. In addition, the conversion of adenine to guanine for incorpora-
tion into RNA guanine must be inhibited in the cells grown in the absence of inositol, since the difference between the specific activities of RNA adenine and RNA guanine was much greater in the deficient cells than in the normal cells (Fig. 6). Thus the concerted action of these two effects of inositol deficiency would lead to a decrease of the cellular RNA and inhibition of cell multiplication.

The present finding that inositol in some way affects the biosynthesis of nucleotides, and especially of guanine nucleotides, could explain the observations of Ghosh et al. (1) that inositol is necessary for the normal biosynthesis of mannan and glucan. The participation of nucleotides in the biosynthesis of polysaccharides such as glucan, glycogen, cellulose, and others has been demonstrated by many investigators (17), and it is tempting to suggest that guanine nucleotides may be involved in the biosynthesis of yeast mannan. Thus the inability of inositol-deficient cells to synthesize adequate amounts of guanine nucleotides may represent the basic biochemical aberration characteristic of inositol deficiency and common to both yeast and mammalian cells.

**SUMMARY**

The effect of inositol deficiency on the metabolism of nucleic acids has been studied with KB cells. It was shown that inositol deficiency causes a marked decrease in the amount of the acid-soluble nucleotides and the ribonucleic acid of the cells. No effect was observed on the cellular content of deoxyribonucleic acid and protein.

The rate of incorporation of C14 from uniformly labeled glucose-C14 into pool nucleotides and into ribonucleic acid adenine, cytosine, and uracil of the deficient cells proceeded at a rate similar to that obtained with normal cells. However, the rate of labeling of ribonucleic acid guanine in the deficient cells was half of that of normal cells.

The rate of labeling of the deoxyribonucleic acid bases was uniformly decreased in the inositol-deficient cells and reflected the decreased rate of cell multiplication.

Evidence is presented which suggests that only a small fraction of the pool guanine participates in the biosynthesis of ribonucleic acid guanine.

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**REFERENCES**

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