Simultaneous Measurement of RNA, DNA, and Protein Synthesis in Mouse Tumor and Reticuloendothelial Tissue Slices Using Glucose-6-\(^3\)H as a Common Precursor

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SUMMARY

The need for an in vitro cytotoxic drug sensitivity test covering a broad metabolic field has led to the development of a method for simultaneous assessment of RNA, DNA, and protein synthesis in vitro by mouse tumor and reticuloendothelial tissue slices using glucose-6-\(^3\)H as a common precursor. \(^3\)H/\(^1\)4C ratios are given following incubation with both glucose-6-\(^3\)H and glucose-6-\(^1\)4C, and the time course of \(^3\)H incorporation into protein and the nucleic acids by slices of mouse spleen, thymus, lymph nodes, and tumor is presented. The use of actinomycin D and puromycin shows that incorporation into protein, RNA, and DNA can be separately measured on the same sample. This is further supported by detailed examination of the hydrolysis products of protein and the nucleic acids. Results are discussed in relation to other methods of measuring nucleic acid and protein biosynthesis and to the known pathways of anabolic conversion of glucose to protein and nucleic acids.

INTRODUCTION

The investigations described in this paper were undertaken preliminary to the design of a sensitivity test aimed at measuring in vitro the relative effects of cytotoxic drugs on reticuloendothelial and tumor tissues in mice and humans. Since the site or mechanism of action of many such drugs, particularly the alkylating agents, is not fully understood (33), it appeared important to utilize a common precursor, the metabolism of which would cover a wide range of the reactions, particularly those involved in the biosynthesis of protein, RNA, and DNA. The technic of using a common precursor, in addition to covering a large area of metabolism, could also enable precursor incorporation to be measured simultaneously into these compounds on the same tissue sample, an important factor when dealing with small quantities of tissue such as mouse lymph nodes or human biopsy samples.

Glucose labeled at the carbon-6 position was selected as the common metabolic precursor, and the present study examines the use of the tritium-labeled compound, glucose-6-\(^3\)H, for incorporation in mouse tumor and reticuloendothelial tissues.

MATERIALS AND METHODS

Materials

Glucose oxidase and peroxidase were obtained in a “Biochemica Test Combination” for blood sugar from the Boehringer Corporation Ltd., (London, W.5.). The DNA (type 1), RNA (type X1), deoxyribonuclease 1 (DN-C), and the deoxyribonucleoside standards were all obtained from Sigma Chemical Co. (London, S.W.6.). Mylase P (obtained from A. oryzae) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., England.)

Glucose-6-\(^1\)4C, glucose-6-\(^3\)H, and leucine-\(^1\)4C were obtained from The Radiochemical Centre (Amersham, Bucks.).

Actinomycin D was obtained from Merck, Sharp & Dohme Inc. (Rahway, N. J.) and puromycin dihydrochloride from Nutritional Biochemicals Corp. (Cleveland, Ohio).

All other chemicals used in the investigation were obtained from British Drug Houses Ltd. (Poole, Dorset).

Animals and Tumor

Young adult female mice from inbred colonies of NIH strain (Scientific Animal Services, Elstree, Herts.) or DBA.2J strain (Jackson Laboratory, Maine) were used. The tumor was an isologous implanted mammary adenocarcinoma (CaD\(_2\)) main tained in the strain of origin (DBA.2J) by serial solid transfer. Experiments were carried out approximately 11 days after implantation when the tumor weighed 300–500 mg.

Tissue Preparations and Incubation

Mice were killed by cervical fracture; the tissues were rapidly dissected and dropped into ice-cold Krebs-Ringer phosphate medium, pH 7.3 (32) containing 0.01% unlabeled glucose. When lymph glands were studied, the axillary pair were taken from each side of the animal. When necessary (lymph glands and thymus) the tissues were freed of contaminating fat and connective tissue with the aid of a dissecting microscope. Tissues were blotted to remove excess moisture, rapidly weighed, and then sliced mechanically in a tissue chopper based on that
of McIlwain and Buddle (22) set to slice at 0.5-mm intervals. The sliced tissues were transferred to 25-ml conical flasks containing 1 ml of ice-cold Krebs-Ringer medium. Except where otherwise stated, not more than 40 mg wet weight tissue were taken per incubation flask. In the case of lymph glands, the quantity used could be as little as 8 mg per flask. A further 1-ml sample of the medium, containing 5 μc (0.01 μmoles) of glucose-6-3H, was then added unless otherwise indicated in the tables of the Results section. The flasks were incubated in a shaking water-bath at 37°C for the required times. At the end of incubation, flasks were cooled in crushed ice, and 1 ml of an ice-cold 10% aqueous solution of unlabeled glucose was added.

Separation of RNA, DNA, and Protein

The standard procedure used for the experiments (except where otherwise stated) was partly based on the method of Ogur and Rosen (24). The details are as follows. The tissue and medium were transferred to a small hand-operated homogenizer with a nylon sphere pestle (30) of 10 ml capacity. After brief homogenization the mixture was transferred to a 15-ml conical centrifuge tube, and 1.5 ml of ice-cold 10% (w/v) perchloric acid was added. The precipitate containing protein, lipids, and nucleic acids was centrifuged and washed by four successive resuspensions and centrifugations in 3 ml of ice-cold 2% (w/v) perchloric acid. Lipids were extracted by successive washes in 1 ml ethanol, 2 ml chloroform:ethanol (1:1) twice, and finally 1 ml ethanol. After carefully draining the tubes of ethanol, the precipitate was resuspended in 1 ml ice-cold N perchloric acid and left for 18 hours at 4°C to extract RNA. The tube was then centrifuged, the extract set aside, and the precipitate resuspended in 0.5 ml cold N perchloric acid and recentrifuged. The supernatant was combined with the first extract. For extraction of DNA, the precipitate was resuspended in 1 ml 0.5 N perchloric acid and heated in a water bath at 70°C for 20 min. The extract obtained by centrifugation was set aside, and the precipitate was reextracted with 0.5 ml 0.5 N perchloric acid at 70°C for 20 min. The extracts were combined. The residual protein precipitate was washed twice with 2 ml distilled water and then dissolved in 1 ml KNO3 by warming at 50°C for 1 hour.

Determination of Specific Radioactivity of RNA, DNA, and Protein

The RNA and DNA extracts were neutralized with a few drops of 10 N KOH, and, after cooling, the precipitated potassium perchlorate was removed by centrifugation. The absorption spectra were measured in a Unicam SP 800 spectrophotometer (Unicam Instruments Ltd., Cambridge, England) and were found to correspond closely to the spectra of the purified commercially obtained DNA and RNA standards measured under similar conditions. In practice it was found convenient to measure the extinction at 260 μm (1 cm path length) of 0.3 ml aliquots of the RNA extracts diluted with 3.0 ml distilled water and to compare this with a known amount of RNA standard. DNA was determined similarly at 270 μm. Another aliquot (0.6 ml) of neutralized RNA or DNA extract was transferred to a counting vial for determination of radioactivity as described below. Protein content was determined by the following modification of the method of Lowry et al. (21). An 0.1-ml sample of the protein dissolved in N KOH was taken, and 6 ml of 2% (w/v) Na2CO3 containing 0.01% CuSO4, 5H2O and 0.02% potassium tartrate were added. After 10 min, 0.1 ml of diluted (1:1) Folin and Ciocalteu’s reagent (21) was added, and the extinction at 750 μm was measured after exactly 30 min. A standard curve was constructed using crystallized bovine albumin (BDH, Poole, Dorset) under these experimental conditions. A further aliquot (0.6 ml) of the dissolved protein was transferred to a counting vial. Radioactivity was determined using a Beckman liquid scintillation spectrometer (Type LS.200) in which quench correction is obtained by ratio of an external standard counted in two channels. Samples were mixed with 16 ml of the following scintillation mixture: 4 g m of 2,5-bis-5-tert-butylbenzoxazol-2'-thiophene (BBOT, CIBA Ltd., Duxford, Camb.), 400 ml 2-methoxyethanol, 80 gm naphthalene (molecular weight determination grade), and 600 ml toluene (A.R.). The samples were then counted twice for 20 min or until 20,000 counts were collected. Correction was made for background, decay, and quenching (using a series of standards prepared at different quenching levels in the same scintillation mixture). This enabled results to be obtained in terms of absolute radioactivity (μμc). The radioactivity was divided by the weight of RNA, DNA, or protein measured as above to give specific radioactivity (μμc/100 μg).

Experiments to Examine the Specific Activities of the Components of RNA, DNA, and Protein

In order to obtain sufficient material of high activity for examination of hydrolys products, the pooled spleens of six mice were used to yield 500 mg of sliced tissue, and this was incubated in 20 ml of incubation medium containing 500 μc of glucose-6-3H (1.0 μmoles) for 60 min. After incubation, 5 ml of 20% (w/v) glucose was added and the sample was cooled and homogenized. Two 1-ml samples were taken for separation of RNA, DNA, and protein by the standard procedure as described above. The remainder was divided into two equal portions. One portion was centrifuged and extracted with M NaCl as described by Wyatt (34) for preparation of purified DNA for enzymic hydrolysis as described below. The other portion was used for extraction and hydrolysis of RNA and also of protein as described below.

Separation of Deoxyribonucleosides

The dried purified DNA was dissolved in 2 ml 0.01 M Tris maleate buffer, pH 6.7, containing 100 μg deoxyribonuclease, 5 mg mylase-P, 15 μmoles MgSO4, and ethylmercurithiosalicylate to a final concentration of 0.01%. This mixture was incubated at 30°C for 20 hours to liberate deoxyribonucleosides from the DNA (5). After incubation, the mixture was deproteinized by emulsification three times with chloroform:octanol (8:1 v/v) (5) followed by centrifugation. The deproteinized hydrolysate was taken to dryness in a vacuum desiccatator at room temperature over P2O5 and then redissolved in
about 0.1 ml water for loading on to a thin-layer chromatogram. Separation of the nucleosides was carried out on a 20 x 20 cm plate coated with cellulose (MN-cellulose powder 300, Macherey, Nagel & Co., Duren, Germany) at a thickness of 0.25 mm. The plate was developed in two dimensions; the first solvent was distilled water and the second was isopropanol:5.7 N HCl (65:35 v/v). The deoxyribonucleosides were located by examining the dried plates under short-wave length UV light and identified by comparing positions with those of standards treated in a similar manner. The spots were then eluted with water and UV absorption spectra measured at pH 2.0. These were compared with the spectra of the standard nucleosides and with published data (10, 29) to verify identification and to give a quantitative estimation of each nucleoside. It was found that deoxyguanosine was decomposed to the free base by the second solvent, but, fortunately, this nucleoside could be separated from the other three in the first solvent (water). The Rf values in water were 0.49 for deoxyguanosine, 0.64 for deoxyadenosine, 0.60 for deoxycytidine, and 0.69 for thymidine. After quantitating each nucleoside, the solution was evaporated to dryness in a vacuum desiccator over P2O5 to remove both water and HCl. The dry residue was dissolved in 0.5 ml water and mixed with scintillation fluid for determination of radioactivity.

**Extraction and Hydrolysis of RNA and Separation of Ribonucleotides**

The homogenized sample was precipitated with perchloric acid, washed, and extracted with lipid solvents as in the standard procedure described above. After the final ethanol extraction, however, the portion used for separation of ribonucleotides was washed twice with ether and the precipitate dried. RNA was then extracted with 10% (w/v) NaCl, purified, and hydrolyzed as described by Graymore (13). The neutralized hydrolysate was subjected to high-voltage paper electrophoresis to separate the ribonucleotides (25). The nucleotide spots were eluted with 2 ml water, and 0.6-ml aliquots were taken for determination of radioactivity as described above. The remainder of the eluate was scanned in a Unicam S.P. 800 spectrophotometer (Unicam Instruments Ltd., Cambridge) to verify and measure the nucleotides by means of their specific absorption characteristics in the UV region (3).

**Hydrolysis of Protein and Separation of Amino Acids**

The protein, after extraction of nucleic acids and lipids, was washed in acetone and ether and air-dried; 2.5 mg dry weight protein were mixed with 10 ml of 6 N HCl and heated in a sealed glass bomb at 110°C for 18 hours. On cooling, the bomb was opened and the contents taken to dryness in a rotary evaporator. The sample was redissolved in 0.1 N HCl for amino acid analysis and fractionation. This was carried out using a Technicon Analyser with the buffer gradient as described by Crawhall et al. (7). In order to obtain amino acids for radioactivity determinations, the column effluent was divided at the bottom of the column so that approximately 80% was collected in a "RadiRac" fraction collector (LKB Instruments Ltd., London) in 2-ml fractions. The remaining 20% was pumped into the optical system of the analyzer via a proportionating pump manifold modified as follows: sample tubing diameter reduced from 0.035 cm (internal diameter) to 0.015 cm, nitrogen tubing reduced from 0.045 cm (internal diameter) to 0.035 cm, ninhydrin tubing diameter unchanged. On completion of the 20-hour analysis, the quantity of each amino acid was calculated from the recording chart and the position of the amino acids in the fractionated effluent ascertained. Samples containing the same amino acid were pooled and 0.8-ml aliquots taken for determination of radioactivity as described above. Aliquots of the effluent not containing amino acid were also taken for radioactivity determination.

**RESULTS**

Preliminary to the use of labeled glucose as common precursor for protein and nucleic acid synthesis in tissue slices, it was necessary to determine whether the overall concentration of glucose in the incubation medium, namely 0.01%, was sufficient for tissue requirements during the incubation period. Several experiments were thus carried out using the glucose oxidase, peroxidase method to determine the rate of disappearance of glucose from the incubation medium in the presence of mouse spleen or tumor slices. It was found that the mean glucose disappearance rate from five experiments in which the tissue quantity per 2-ml incubation medium ranged from 55 to 160 mg (wet weight) was 1.03 ± 0.10 (S.D.) μmoles glucose disappeared/100 mg wet weight spleen/hour. Similar experiments with tumor slices gave values of 0.80 ± 0.03 (S.D.). Therefore, with the glucose concentration in the medium at 0.01% (1.11 μmoles in 2 ml) by limiting the maximum tissue quantity to 40 mg in 2 ml, more than half the glucose remains in the medium after one hour's incubation.

In order to demonstrate further that the glucose concentration in medium did not limit tissue requirements in macromolecular biosynthesis, experiments were carried out in which immediate precursor incorporation into protein was determined at 0.01% glucose and compared with that using a medium containing glucose at 0.10%. L-Leucine-U-14C (1 μc, 19.7 μg per 2 ml medium) was used with 40 mg wet weight of spleen slices. The mean specific activities (μc/100 μg) of protein from six determinations were 1167 ± 36 (S.E.) with the low glucose medium and 1112 ± 50 (S.E.) with the high glucose medium. Thus a tenfold increase in glucose concentration in the incubation medium did not increase the rate of incorporation of amino acid into protein. Experiments were then carried out to compare the specific activities of RNA, DNA, and protein with respect to 3H and 14C from tissue incubated with both glucose-6-3H and glucose-6-14C in order to consider the possibility of increasing lability of the labeled hydrogen atoms during the incubation period. Table 1 shows the results of these experiments. It can be seen that the 3H/14C ratios in RNA and DNA do not alter significantly during the last 40 min of the incubation but that the ratio falls slightly when measured in the protein of spleen slices.

The time course of incorporation into RNA, DNA, and protein was then studied in slices of three tissues of the reticuloendothelial system. The results using thymus, spleen, and axillary lymph nodes are shown in Table 2. It can be seen that
Table 1

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>RNA</th>
<th>Spleen</th>
<th>Tumor</th>
<th>DNA</th>
<th>Spleen</th>
<th>Tumor</th>
<th>Protein</th>
<th>Spleen</th>
<th>Tumor</th>
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</thead>
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<tr>
<td>20</td>
<td>4.87 ± 0.26</td>
<td>4.79 ± 0.66</td>
<td>4.22 ± 0.39</td>
<td>4.58 ± 0.24</td>
<td>3.57 ± 0.14</td>
<td>3.63 ± 0.05</td>
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<tr>
<td>40</td>
<td>4.94 ± 0.26</td>
<td>4.78 ± 0.10</td>
<td>3.97 ± 0.10</td>
<td>4.05 ± 0.08</td>
<td>3.26 ± 0.11</td>
<td>3.41 ± 0.11</td>
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<tr>
<td>60</td>
<td>4.61 ± 0.20</td>
<td>4.43 ± 0.17</td>
<td>3.98 ± 0.20</td>
<td>4.10 ± 0.10</td>
<td>3.16 ± 0.14</td>
<td>3.34 ± 0.03</td>
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Table 2

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>RNA</th>
<th>Spleen</th>
<th>Lymph nodes</th>
<th>DNA</th>
<th>Spleen</th>
<th>Lymph nodes</th>
<th>Protein</th>
<th>Spleen</th>
<th>Lymph nodes</th>
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<tr>
<td>15</td>
<td>39 ± 9</td>
<td>17 ± 2</td>
<td>54 ± 16</td>
<td>41 ± 2</td>
<td>24 ± 2</td>
<td>27 ± 4</td>
<td>18 ± 5</td>
<td>20 ± 5</td>
<td>49 ± 6</td>
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<tr>
<td>30</td>
<td>117 ± 27</td>
<td>94 ± 16</td>
<td>207 ± 8</td>
<td>72 ± 8</td>
<td>69 ± 9</td>
<td>156 ± 20</td>
<td>58 ± 7</td>
<td>59 ± 5</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>45</td>
<td>130 ± 13</td>
<td>130 ± 13</td>
<td>269 ± 10</td>
<td>90 ± 14</td>
<td>91 ± 9</td>
<td>212 ± 13</td>
<td>121 ± 10</td>
<td>71 ± 3</td>
<td>213 ± 19</td>
</tr>
<tr>
<td>60</td>
<td>197 ± 15</td>
<td>168 ± 15</td>
<td>231 ± 18</td>
<td>162 ± 10</td>
<td>162 ± 10</td>
<td>225 ± 49</td>
<td>145 ± 7</td>
<td>132 ± 12</td>
<td>167 ± 4</td>
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<tr>
<td>75</td>
<td>195 ± 17</td>
<td>227 ± 21</td>
<td>223 ± 42</td>
<td>167 ± 17</td>
<td>209 ± 13</td>
<td>255 ± 24</td>
<td>133 ± 20</td>
<td>145 ± 14</td>
<td>223 ± 9</td>
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Time course of $^3$H incorporation into RNA, DNA, and protein by mouse thymus, spleen, and lymph node slices incubated with glucose-6-$^3$H.

aMean ± S.E. of 4 determinations for each result.

Table 3

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>RNA</th>
<th>Spleen</th>
<th>Lymph nodes</th>
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<tr>
<td>15</td>
<td>122 ± 5</td>
<td>216 ± 17</td>
<td>82 ± 10</td>
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<tr>
<td>30</td>
<td>296 ± 23</td>
<td>470 ± 36</td>
<td>179 ± 11</td>
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<td>45</td>
<td>588 ± 40</td>
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<td>60</td>
<td>765 ± 36</td>
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</tr>
<tr>
<td>75</td>
<td>827 ± 42</td>
<td>1090 ± 48</td>
<td>593 ± 20</td>
</tr>
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</table>

Time course of $^3$H incorporation into RNA, DNA, and protein by mouse tumor slices incubated with glucose-6-$^3$H.

aMean ± S.E. of 5 determinations for each result.

Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA</th>
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<tr>
<td>None</td>
<td>139 ± 9</td>
<td>163 ± 11</td>
<td>159 ± 13</td>
</tr>
<tr>
<td>Actinomycin D 2 μg/ml</td>
<td>162 ± 6</td>
<td>112 ± 14</td>
<td>179 ± 10</td>
</tr>
<tr>
<td>Actinomycin D 10 μg/ml</td>
<td>135 ± 4</td>
<td>44 ± 6</td>
<td>181 ± 21</td>
</tr>
<tr>
<td>Puromycin 5 μg/ml</td>
<td>96 ± 3</td>
<td>175 ± 18</td>
<td>173 ± 21</td>
</tr>
<tr>
<td>Puromycin 50 μg/ml</td>
<td>36 ± 4</td>
<td>156 ± 22</td>
<td>156 ± 11</td>
</tr>
</tbody>
</table>

Effects of actinomycin D and of puromycin on incorporation of $^3$H into protein, RNA, and DNA during incubation of mouse spleen slices with glucose-6-$^3$H. Incubation time was 60 min. The pooled spleens of 12 mice were used.

aMean ± S.E. of 6 determinations for each result.
with that of the standard procedure. The specific activities of ribonucleotides from alkaline hydrolysis of RNA, and deoxyribonucleotides from enzymic hydrolysis of DNA, are shown in Table 6. The results show that the purine-based ribonucleotides are more highly labeled than the pyrimidine nucleotides and that adenylic acid in particular is highly labeled. Following fractionation of DNA, the deoxyribonucleoside with the highest specific activity is thymidine while deoxyadenosine labeling is low. Also from the data of radioactivities of the ribonucleotides and quantity of each present in the RNA hydrolysate, it is possible to calculate a value for specific activity of RNA to which only the four nucleotide components have contributed. This value is given in Table 6 and agrees reasonably with the value for RNA as obtained by the standard procedure. A similar value is also calculated for DNA and again shows good agreement with the original DNA. Acid hydrolysis of protein from such experiments was also carried out followed by fractionation into the constituent amino acids. Table 7 shows the specific activities from a typical experiment. It may be seen that only four amino acids are highly labeled, serine, glutamic acid, proline, and alanine; of these serine is by far the most active. A calculated value of specific activity for the protein as obtained from the amino acid values is also given and agrees well with that of the unhydrolyzed starting material.

**DISCUSSION**

The present study has arisen from the need for an in vitro test system with two main requirements. First, a measure of incorporation into RNA, DNA, and protein was required using small quantities of tissue. Assessment on a single sample of two of these parameters could be achieved by the use of double-labeling technics, but not of all three. The second requirement was for a system covering a broader field of metabolic pathways for nucleic acid biosynthesis than provided for by the use of labeled nucleosides. It has been shown that certain fluoropyrimidine antimetabolites, while inhibiting DNA synthesis and mitosis in mammalian cells (2), do not inhibit incorporation of thymidine (6), the most widely used precursor for DNA synthesis studies. These findings suggested a block in the de novo synthesis of DNA which could not be detected by the use of labeled thymidine. Since studies are planned using such antimetabolites and also cytotoxic agents whose mechanism of action is unknown, it seemed unwise to limit the scope and accuracy of such studies by the use of labeled nucleosides. Labeled glucose appeared to offer a more physiologic approach to the de novo synthesis of nucleic acid precursors while at the same time providing a measure of incorporation of some amino acids into protein.
In studies on the utilization of glucose-6-\(^{14}\)C for synthesis of ribose and deoxyribose of rat tissue nucleic acids in vitro (16, 17), it was shown that incorporation in liver slices was much lower than in thymus or spleen. This was ascribed to extensive glycogenolysis during incubation, with a resultant dilution of labeled glucose. The technic described in the present study is thus not suitable for a tissue such as liver with large stores of glycogen but is ideally suited to reticuloendothelial tissues and to tumor tissues, known to have a very low glycogen content (23). Care must be taken, however, not to limit the availability of glucose in the medium, and this has been achieved by allowing an ample margin of glucose in the medium over that disappearing during the incubation period.

The use of glucose-6-\(^{3}\)H as opposed to glucose-6-\(^{14}\)C was prompted by the much lower cost of the \(^{3}\)H derivative. This could become an important factor when considering the use of large numbers of samples necessary to produce statistically meaningful results in the testing of cytotoxic drugs. Using this isotope could, however, present some problems that must be considered. A loss of tritium relative to carbon has been observed in the intact rat after administration of both types of labeled glucose (9) and a possible metabolic cleavage of the C-H bond at C-6 suggested. In rat liver and diaphragm slices in vitro, however, Bloom and Foster (2) found that no tritium was lost from C-6 of glucose formed intracellularly from double-labeled glycerol. Examination in the present study of carbon/tritium ratios in protein and the nucleic acids after incubating spleen slices showed the expected loss of tritium in protein as a result of dehydrogenation steps in the citric acid cycle. In addition, however, the protein \(^{3}\)H/\(^{14}\)C ratio fell by about 10% during the last two-thirds of the incubation period, indicating a small loss of tritium. The ratio was more constant in the nucleic acids, suggesting that the relative loss of tritium did not occur at the level of glucose or glucose-6-phosphate but at some later stage in the metabolic pathway not common to all three end-products. The higher ratios in RNA compared with DNA observed throughout the incubation are hard to explain, and the possibility of an isotope discrimination effect cannot be completely ruled out. Such effects have been observed in the metabolism of tritiated glucose (19), although the main effects appeared to be directed at the C-1 position and not at C-6. The results of the present study, however, would suggest that if any isotope discrimination effect is present it is directed at some later metabolite, possibly a precursor of RNA.

The somewhat similar rates of progress of incorporation into RNA and DNA of the three tissues of the reticuloendothelial system examined accord in general with the in vivo studies of Itzhaki and Whittle (18), who showed that labeling rates of ribose and deoxyribose in rat spleen and thymus were similar after injection of glucose-6-\(^{14}\)C. In studies in vitro, however, (17) they found that, after three hours incubation under oxygen, the relative specific activity of thymus RNA ribose was higher than that of spleen. In the present studies the incorporation into spleen continued to increase after thymus had reached a plateau. Since, however, the incubation was carried out under air and not oxygen, survival of metabolic functions may be prolonged by the presence of erythrocytes in the spleen slices. The relatively higher statistical variation in results with lymph node nucleic acids reflects some of the problems inherent in the use of very small quantities of tissue consisting of a heterogeneous mixture of cells of widely differing metabolic activities. Incorporation by tumor slices was considerably higher than by slices of reticuloendothelial tissue; specific activity of protein was approximately three times and nucleic acids up to five times as high. This result may be expected since the implanted tumor is a rapidly dividing and growing tissue. Measurement of tumor growth rate at the time the mice were killed showed tumor weight to be increasing by 27% of the wet weight each day.

Reich et al. (26) showed that actinomycin D inhibits RNA but not DNA synthesis of mammalian tissue cell cultures. They further showed that protein biosynthesis is not suppressed. The block in RNA synthesis has been ascribed to specific inhibition of DNA-dependent RNA polymerase (12) and may prove to be related to the formation of a complex between actinomycin D and DNA (27). In the present study, actinomycin D only inhibited incorporation into RNA, evidence that no gross contamination with RNA occurred in the DNA and protein fractions. The concentrations of the drug required to produce inhibition were, however, somewhat higher than is sometimes observed with tissue slices. Thus, Ilan et al. (15), studying the effects on beetle pupa slices, obtained 80% inhibition with actinomycin D at 1.6 \(\mu\)g/ml, and Dickman and Yang (8) used 0.5 \(\mu\)g/ml for inhibition in dog pancreas slices. However, both the mouse spleen slices and the tumor studied here have a high DNA content, and, since DNA has been demonstrated to protect against the inhibitory action of actinomycin D (11), more actinomycin may be required to produce the same inhibition in lymphoid and tumor tissue slices than in tissues with relatively less DNA.

Puromycin is now widely accepted as a specific inhibitor of protein synthesis, acting by premature release from the ribosomes of incomplete polypeptide chains (1). The 74% inhibition of incorporation into protein by concentrations which did not affect incorporation into RNA and DNA is further proof of a satisfactory separation in the present study.

The most direct evidence that incorporation into protein, RNA, and DNA can be separately assessed using glucose-6-\(^{3}\)H as a common precursor comes from an examination of the hydrolysis products. In the case of the amino acids, labeling was very low in all the essential amino acids. Any labeling of these amino acids could be explained by the inaccuracies of counting the very low activity samples obtained from the amino acid fractionation. Of the nonessential amino acids measured, aspartic acid and glycine had very little activity, whereas serine was very highly labeled. This can be explained by consideration of the metabolic pathways leading to the synthesis of these amino acids. Ichihara and Greenberg (14) reported formation of serine from glyceric acid and 3-phosphoglyceric acid in mammalian tissues. Present results suggest a high rate of synthesis by these routes in spleen slices. The subsequent conversion of serine to glycine involves the removal of the \(\beta\)-carbon and its hydrogen atoms (31), thus explaining the lack of labeling of glycine in the present study.

Aspartic acid, synthesized by transamination from oxaloacetic acid, could be expected to be unlabeled due to removal of labeled hydrogen in the succinic and malic dehydrogenase steps of the citric acid cycle, whereas glutamate is expected to
be labeled, arising as it does from α-ketoglutarate, in which the tritium-carbon bond is still intact. Both these predictions are verified by present experiments.

On examination of the RNA hydrolysis products, it was noted that the pyrimidine nucleotides were labeled less than the purine nucleotides. Itzhaki and Whittle (17), using glucose-6-14C as a precursor for RNA ribose in vitro, showed very similar results. In the present studies, however, consideration must be given to any possible contribution due to labeling of the bases. The pyrimidine skeleton is synthesized from carboxymethylphosphate and aspartic acid (4). Since aspartic acid labeling is very low, it is unlikely that the cytidylic and uridylic acids of RNA are labeled with anything other than ribose. In the case of purine synthesis, however, C-2 and C-8 and their associated H-atoms are derived from the β-carbon of serine (28), which is very highly labeled in the present experiments. This could, therefore, contribute to the overall labeling of adenylc and guanylic acids. The relatively lower specific activity of guanylic acid could also be explained on this basis since in guanine the labeled hydrogen on C-2 of the purine skeleton would be replaced by an amino group.

Similar considerations may be applied to the DNA hydrolysates. Thus the relatively high labeling of thymidine could be explained by the contribution made to the pyrimidine moiety of the methyl group on C-5, a major source of which is again the β-carbon of serine (4). The low activity of deoxyadenosine in the DNA hydrolysate is difficult to explain and may indicate partial destruction of this nucleoside in the chromatographic procedure, as was observed with deoxyguanosine.

The values of specific activity calculated for RNA, DNA, and protein from the relative contributions of their components agree with the specific activity as measured by the standard procedure adopted in this investigation. This further supports the view that labeling is free from artefact and represents the in vitro anabolic conversion of glucose to protein and nucleic acids.

It is believed that the method described can form the basis of an in vitro test of broad application to the selection of cytotoxic drugs for cancer chemotherapy and also act as a starting point for the elucidation of mechanisms of action of such drugs.

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