Autoradiography of Diffusible Compounds in Human Nonnucleated Erythrocytes: Studies with Tritiated Glucose and Adenine

By Tibor J. Greenwalt, Joseph C. Paige, Nobukoh Etoh, and Edwin A. Steane

Autoradiography has been used extensively for the localization of labeled substances in microscopic structures. Its applicability has been restricted mainly to compounds which are firmly bound and are not leached or translocated by histologic and histochemical processing, e.g., thymidine incorporation into DNA. The inapplicability of autoradiography to the study of substances which remain diffusible after conventional fixation of tissue sections and smears has taxed the ingenuity of many investigators and the first conference devoted to this problem was recently published.

The technical difficulties and the importance of being able to prepare useful autoradiograms of diffusible substances in erythrocytes are not immediately apparent even to those who are expert with the well-established and relatively simple techniques used for autoradiography of compounds which are readily immobilized. With soluble, diffusible metabolites it is necessary to prevent diffusion until the final step of photographic development of the nuclear emulsions has been reached. Autoradiographic studies of nonnucleated erythrocytes have therefore been limited to the incorporation of labeled amino acids into proteins by reticulocytes, the distribution of 14C-cholesterol in the membrane lipoproteins and the attachment of antibodies.

The ability to identify differences in the utilization of substrates by single erythrocytes has advantages over quantitative measurements which give only the average values for the total population. It is important to know whether polymorphic traits are expressed uniformly in all cells or if they are present in only a proportion.

The purpose of this paper is to present a modification of a technique for autoradiography of soluble compounds in nonnucleated red blood cells previously reported in brief by the authors. The manner in which the procedure was used to study the metabolism of glucose-6-3H and adenine-8-3H is also described. The results permit answers to such questions as: Is the decreased

---

Contribution No. 184 from the Blood Research Laboratory, American National Red Cross.
Presented in part at the 53rd meeting of the Federated Societies of Experimental Biology, April 16, 1969, Atlantic City, and the XIIth Congress of the International Society of Blood Transfusion, August 19, 1969, Moscow, U.S.S.R.
Supported by USPHS Grant HE-11154 from the National Heart and Lung Institute.
Tibor J. Greenwalt, M.D.: Medical Director, Blood Program, American National Red Cross and Clinical Professor of Medicine, George Washington University School of Medicine, Washington, D.C.; Joseph C. Paige, M.S.; Nobukoh Etoh, B.S.; and Edwin A. Steane, B.S.: Research Technologists, American National Red Cross.
utilization of glucose during storage of ACD blood uniform in all the red blood cells? Does added inosine affect the incorporation of adenine differently in fresh red blood cells and in stored red blood cells? Does incubation at 37°C have a favorable or an unfavorable effect on the incorporation of adenine?

**Materials and Methods**

**Blood Samples**

The donors were healthy male and female staff members. A. *Glucose utilization studies.* Blood samples were collected with acid–citrate–dextrose (ACD), NIH formula B (Becton-Dickinson and Co. yellow-top vacutainers, S3204X). B. *Adenine incorporation studies.* Blood samples were collected into ACD, NIH formula A with a final concentration of 0.5 μmoles adenine/ml in the blood-preservative mixture (ACD-adenine solution kindly supplied by Dr. William Warner, Baxter Laboratories, Morton Grove, Ill.).

**Buffers and Reagents**

*Tsutai’s buffer.* Glucose 5 mM., NaH₂PO₄ 0.025 M, glycine 0.05 M, NaCl 0.05 M, adjusted to pH 8.2 with 1.0 N NaOH. This solution was sterilized by autoclaving in 60-ml stoppered flasks.

*Buffered saline (PBS).* Hendry’s buffered saline pH 7.4 was used.

*Nuclear emulsion.* Approximately 30 ml. of Ilford K-5 nuclear emulsion gel was melted without dilution in a beaker placed in a 45°C water bath in the darkroom. Unused emulsion from each experiment was discarded. The film for overlaying the blood smears was formed by dipping a circular wire loop 3.5 cm. in diameter into the emulsion. Nickel–chromium alloy, size 22 wire was used for making the loops which were taped to glass slides for ease of handling.

*Developing and fixing solutions.* Stock developer solution was prepared by dissolving 20 oz. of Dektol (Eastman Kodak) in 3.8 liters of distilled water at 38°C. For use, the stock solution was diluted with an equal volume of distilled water. Fixing bath was prepared so that one liter of distilled water contained 240 Gm. Na₂S₂O₃·5H₂O, 15 Gm. dessicated Na₂SO₃, 48 ml. of 28 per cent acetic acid, 7.5 Gm. H₃BO₃ crystals and 15 Gm. AlK(SO₄)₂·12 H₂O.

*Preparation of Autoradiograms*  
Contact smears of the washed red blood cells were pulled at the ends of 1 × 3 inch glass slides and dried rapidly at 60°C by placing on a hot plate under a current of warm air from a hair dryer. Working in a darkroom, with a Kodak safelight using a Wratten series 2 filter and a 15-watt bulb as the only light source, the blood smears were overlaid with the film of Ilford K-5 emulsion. The slides were stored at 4°C in black plastic boxes sealed with electrician’s black tape. A small packet of Drierite wrapped in tissue paper was placed in each box to prevent the condensation of moisture.

After the required period of exposure, the slides were transferred to staining racks in the darkroom and immersed for 10 minutes in developing solution chilled to 4°C to permit even penetration of the solution through the emulsion. The slides were transferred for two minutes to another bath of developer kept at 13–15°C. Gentle washing in water for 30 seconds was followed by five minutes in the bath of fixative at 13–15°C. The slides were allowed to dry at room temperature after a final 10-minute rinse in running tap water.

*Counting of Grains*  
No counterstain or coverslips were used. The grain distribution in 1000 red blood cells in each sample was determined by light microscopy under oil immersion. The slides were coded and counted “blind” to avoid subjective bias. Only grains lying on or in direct contact with a red cell were counted as intraerythrocytic. The number of free grains were
also counted to permit calculation of the percentage of the total grains associated with red blood cells. By recording the number of oil immersion fields required to enumerate 1000 corpuscles and having determined that 550 red cells were needed to fill one microscope field it was possible to determine the actual distribution of the grains in any autoradiogram. The formula used was:

\[
\frac{\text{Total number of red cells counted}}{(\text{No. of microscopic fields used } \times 550) - \text{total no. of red cells counted} \times \frac{\text{No. free grains}}{\text{Total grains}}} \times 100 = \text{corrected per cent free grains.}
\]

The percentages of intra- and extracorpuscular grains with tritiated glucose are given in Table 1 and the data obtained with tritiated adenine are given in Table 2.

**Measurement of Radioactivity in Supernatant Solutions**

The supernatant solutions from some experiments were collected and the radioactivity quantitated in a Packard Auto-Gamma Spectrometer (Model 5019) using 1-4-bis-(5-phenyloxazolyl)-benzene (POPOP) as the phosphor. This was done to monitor the efficiency of the washing steps and to give indirect estimates of the amount of labeled compounds incorporated by the red blood cells. Direct counts of the radioactivity in the red cells was not possible because we were unable to solubilize the glutaraldehyde-treated corpuscles even by vigorous sonication, repeated freezing and thawing, or by the action of Nuclear Chicago "Solubilizer."14

**Protocol for Study of Glucose Utilization**

Four tubes of ACD blood (27 ml.) were collected from each subject. One sample was studied on the day of collection and the other three were stored at 4°C for two, four and five weeks. An aliquot of each sample stored for five weeks was incubated in the presence of 15 mM adenosine15 as a further control to establish the dependence of glucose utilization on the metabolic integrity of the erythrocyte.

The plasma and buffy coat of each sample were removed after centrifugation at 700 g for five minutes and 2.5 ml. of Tsuboi’s buffer was added to 1.0 ml. of the centrifuged red blood cells. One-half milliliter of the resulting red cell suspension and 100 μCi. of glucose-6-3H (2.3 Ci./mmole, Amersham/Searle, Chicago) diluted in Tsuboi’s buffer were placed in a 50-ml Erlenmeyer flask, covered with parafilm and shaken at 90 opm. in a Dubnoff metabolic water bath at 37°C for two hours. A control test run with each sample was treated in the same manner except that 0.5 ml. of Tsuboi’s buffer instead of the tritiated glucose solution was added to the red blood cell suspension.

One-half milliliter of the test and control samples were transferred to 13 × 100-mm. tubes and washed twice with Tsuboi’s buffer allowing the tubes to stand for 10 minutes at room temperature with each wash before centrifuging at 700 × g for three minutes. The washed red cell buttons were then resuspended in 3.5 ml. of 0.5 per cent glutaraldehyde in PBS. After standing at room temperature for 30 minutes, three further washings were performed with PBS after centrifuging and removing the supernatant glutaraldehyde solution. Autoradiograms were then prepared from each test and control sample of red blood cells.

**Protocol for the Study of Adenine Utilization**

Twenty milliliters of blood from each volunteer was collected in 3 ml. of ACD, NIH formula A with sufficient adenine to give a final concentration of 0.5 mM in the blood anticoagulant mixture. Fifteen milliliters of the ACD adenine blood was centrifuged and 2 ml. of plasma removed and replaced with 2 ml. of a sterile solution of adenine-8-3H, 0.88 mCi. (12.3 Ci./mmole, Schwarz BioResearch, Inc., Orangeburg, N. Y.) with 42 mg. of glucose/ml. One-milliliter aliquots of all samples were transferred aseptically to stoppered test tubes.

Two fresh 1-ml. aliquots of blood from each subject were incubated with adenine-8-3H at once for one hour; inosine (Schwarz BioResearch, Inc.) in a final concentration of
It

Fig. 1.—Autoradiograms of glucose utilization studies × 2500. (A) Control of ACD blood stored five weeks and incubated for one hour with 15 μmoles of adenosine per milliliter and no tritiated glucose. Controls had very few silver grains. (B) Erythrocytes from fresh ACD blood incubated for two hours with glucose-6-3H. (C) Red blood cells from ACD blood stored for five weeks and incubated for two hours with glucose-6-3H. Note crenated spheres and paucity of grains. (D) Same as (C) incubated with adenosine as in (A). Crenated spherocytes replaced by corpuscles with central clear zones.

10 mM. was added to one sample. A control sample containing no radioactive adenine was processed in an identical fashion.

All the other samples prepared were stored at 4°C and tubes were removed for study at 1, 7, 14, 21, 35 and 42 days. Duplicate tubes of the 35- and 42-day samples were also subjected to incubation at 37°C for one hour in the presence of 10 mM. inosine.

In preparation for autoradiography following storage or incubation with adenine or adenine plus inosine, 0.5 ml. of each sample was washed three times with PBS, treated with glutaraldehyde as described above and subsequently washed five more times with PBS before making the smears.

Random cultures prepared in thioglycollate medium during the course of all these experiments showed no detectable contamination.

Controls

Control samples from each blood specimen studied were treated in an identical manner as the test samples, except for omission of the tritiated glucose or the tritiated adenine. This included the samples incubated with adenosine and inosine. The efficiency of the washing routine for removing extracellular radioactivity was tested by washing the red cells immediately after adding tritiated glucose or tritiated adenine. The nonradioactive controls never contained more than very occasional grains, and the radioactive controls washed immediately contained single grains associated with 10–30 per cent of the red blood cells (Figs. 1 and 2). Slight hemolysis was observed in the wash solutions at 35 and 42 days of storage.
RESULTS

Various compounds known to react with the red cell membrane and to alter permeability, and some which might form less-soluble complexes with hexose and purine compounds inside the red cells were tried alone and in various combinations. They were all found useless or definitely inferior to glutaraldehyde and therefore will only be listed for the negative value of the record: CaCl₂, BaCl₂, CuCl₂, NaF, Na₂B₄O₇, formaldehyde, tannic acid, osmic acid, ouabain, ethacrynic acid, parachloromercuribenzoate, parachloromercuribenzenesulfonate, 1-fluoro-2, 4 dinitrobenzene and 1,5-difluoro-2,4 dinitrobenzene.

Many preliminary studies were made to find the optimal conditions for performing the studies with tritiated glucose. A sample of the original glucose-6-³H solution and supernatant solutions from each step of the procedure being tested were counted in the liquid scintillation system. The total counts in each sample made it possible to estimate the percentage of radiolabeled material retained in the red cells after each manipulation. In 19 studies the two washes with Tsuboi’s buffer containing “cold” glucose removed an average of
Table 1.—Summary of Data Obtained with Glucose-6-\(^3\)H*

<table>
<thead>
<tr>
<th>Days Stored at 4°C</th>
<th>0</th>
<th>1–3</th>
<th>4–6</th>
<th>7–9</th>
<th>(\geq 10)</th>
<th>No. Grains in 5000 RBC</th>
<th>Per Cent Extracorporeal Grains</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5 ± 0.9</td>
<td>13.8 ± 14.4</td>
<td>32.7 ± 17.7</td>
<td>24.5 ± 10.9</td>
<td>29.5 ± 27.3</td>
<td>34,095</td>
<td>12.3 ± 4.0</td>
</tr>
<tr>
<td>14</td>
<td>1.1 ± 1.2</td>
<td>25.1 ± 22.1</td>
<td>31.2 ± 17.5</td>
<td>17.8 ± 8.5</td>
<td>24.8 ± 28.2</td>
<td>29,982</td>
<td>9.5 ± 4.2</td>
</tr>
<tr>
<td>28</td>
<td>36.6 ± 21.9</td>
<td>50.7 ± 11.5</td>
<td>9.1 ± 11.9</td>
<td>3.2 ± 6.0</td>
<td>0.4 ± 0.8</td>
<td>7,710</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>35a</td>
<td>51.4 ± 15.1</td>
<td>47.2 ± 13.1</td>
<td>1.4 ± 2.1</td>
<td>0</td>
<td>0</td>
<td>3,671</td>
<td>18.7 ± 7.3</td>
</tr>
<tr>
<td>35b</td>
<td>0.3 ± 0.3</td>
<td>9.6 ± 9.7</td>
<td>27.6 ± 18.1</td>
<td>30.2 ± 16.7</td>
<td>32.3 ± 34.9</td>
<td>36,281</td>
<td>9.7 ± 5.3</td>
</tr>
</tbody>
</table>

* 100 \(\mu\)Ci/ml of 15 per cent suspension; 35b incubated one hour at 37°C with 15mM adenosine.
† Mean ± SEM of data from five donors.
P Values calculated by Student's t test: day 0 vs. day 28 (day 0 vs. day 35a, day 35a vs. day 35b) \(\bigg\} \) \(P < 0.01\).
Other comparisons showed no significant differences or are obviously significant.
Table 2.—Summary of Data Obtained with Adenine-8-\textsuperscript{3}H*  

<table>
<thead>
<tr>
<th>Days Stored at 4°C</th>
<th>Incubation One Hour at 37°C</th>
<th>Inosine 10 mM.</th>
<th>Per Cent of Red Cells with Designated Range of Grains</th>
<th>0</th>
<th>1-3</th>
<th>4-6</th>
<th>7-9</th>
<th>≥ 10</th>
<th>No. Grains in 5000 RBC</th>
<th>Per Cent Extracorpuscular Grains</th>
</tr>
</thead>
<tbody>
<tr>
<td>0a</td>
<td>yes</td>
<td>no</td>
<td>3.3 ± 3.9</td>
<td>35.5 ± 25.2</td>
<td>33.3 ± 7.6</td>
<td>21.0 ± 16.5</td>
<td>7.0 ± 7.5</td>
<td>23,750</td>
<td>10.2 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>0b</td>
<td>yes</td>
<td>yes</td>
<td>14.6 ± 8.9</td>
<td>65.5 ± 11.3</td>
<td>18.9 ± 15.2</td>
<td>1.1 ± 2.0</td>
<td>0</td>
<td>10,910</td>
<td>9.4 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>no</td>
<td>no</td>
<td>64.6 ± 11.7</td>
<td>34.9 ± 10.9</td>
<td>0.4 ± 0.8</td>
<td>0</td>
<td>0</td>
<td>2,229</td>
<td>8.1 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>no</td>
<td>no</td>
<td>37.6 ± 14.4</td>
<td>59.7 ± 12.3</td>
<td>2.7 ± 2.2</td>
<td>0</td>
<td>0</td>
<td>5,185</td>
<td>8.7 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>no</td>
<td>no</td>
<td>5.2 ± 3.0</td>
<td>55.4 ± 14.9</td>
<td>32.5 ± 10.6</td>
<td>6.2 ± 6.3</td>
<td>0.8 ± 1.5</td>
<td>16,059</td>
<td>7.8 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>no</td>
<td>no</td>
<td>11.3 ± 5.5</td>
<td>64.7 ± 4.7</td>
<td>21.2 ± 6.3</td>
<td>2.8 ± 3.4</td>
<td>0.1 ± 0.2</td>
<td>14,194</td>
<td>8.6 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>35a</td>
<td>yes</td>
<td>no</td>
<td>4.5 ± 4.9</td>
<td>41.8 ± 11.0</td>
<td>36.8 ± 5.9</td>
<td>14.6 ± 7.5</td>
<td>2.3 ± 2.6</td>
<td>20,131</td>
<td>8.4 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>yes</td>
<td>yes</td>
<td>4.0 ± 4.7</td>
<td>37.5 ± 17.6</td>
<td>38.8 ± 10.8</td>
<td>16.6 ± 10.1</td>
<td>3.1 ± 2.9</td>
<td>21,503</td>
<td>7.3 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>42a</td>
<td>yes</td>
<td>no</td>
<td>9.3 ± 7.6</td>
<td>55.0 ± 6.6</td>
<td>27.8 ± 8.4</td>
<td>7.0 ± 7.0</td>
<td>0.8 ± 0.8</td>
<td>15,237</td>
<td>10.6 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>yes</td>
<td>yes</td>
<td>2.6 ± 1.4</td>
<td>37.2 ± 8.1</td>
<td>37.7 ± 4.1</td>
<td>19.7 ± 5.2</td>
<td>2.8 ± 1.3</td>
<td>22,380</td>
<td>8.1 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

* 69 μCi/ml of ACD adenine blood. The fresh and the 35- and 42-day samples were incubated for one hour with and without the addition of 10 amoles of inosine/ml. Storage was at 4°C after the addition of tritiated adenine.

† Mean ± SEM of data from five donors. Aliquots of blood from five-day samples were incubated for one hour with and without the addition of 10 amoles of inosine/ml. Storage was at 4°C after the addition of tritiated adenine.

\textit{P} values calculated by Student's t test:  
- day 0a vs. day 35a \( P < 0.05; \)
- day 0a vs. day 42a \( P < 0.02; \)
- day 0a vs. day 0b \( P < 0.1; \)
- day 42a vs. day 42b \( P < 0.02. \)

For all other data \( P < 0.1 \) unless stated otherwise in the text.
77.5 per cent ± 8.5 per cent of the radioactivity, leaving 22.5 per cent in the red blood cells.

Glutaraldehyde concentrations of 0.5, 1.0 and 1.5 per cent buffered at pH 6.8, 7.4 and 7.8 with Hendry's buffer were tried. Glutaraldehyde at 0.5 per cent concentration proved the best because only 7.2 per cent (range 5.6–8.0 per cent) of the radioactivity retained after the two washes with Tsuboi's buffer was found in the supernatant solution, whereas the average loss was 18.8 and 29.1 per cent, with one and 1.5 per cent glutaraldehyde, respectively. Variation of the pH as indicated did not have a significant effect and pH 7.4 was selected because the autoradiograms resulting with those preparations appeared to be the best.

**Utilization of Glucose by Red Blood Cells Following Storage**

The distribution of silver grains produced by β-particles originating from the glucose-6-3H metabolized by red blood cells after varying intervals of in vitro storage are summarized in Table 1. There was a time-correlated decline in the ability of the red cells to utilize glucose which was reversed following incubation with adenosine. The total number of grains per 1000 erythrocytes follows a similar pattern and, if day 0 is taken as 100 per cent, the average relative values at 14, 28 and 35 days were 98, 20 and 11 per cent, and after incubation with adenosine at 35 days it rose to 115 per cent. Figure 1 contains photomicrographs of control preparations and autoradiograms of red cells stored in ACD for varying intervals.

**Utilization of Adenine by Red Blood Cells during Storage in ACD-adenine Solution**

The data from the adenine experiments are summarized in Table 2. At 4°C the mean percentage of red cells with more than four grains increased from 0.4 per cent at one day to 53.7 per cent at 35 days, and dropped to 35.6 per cent at 42 days. The total intracorpuscular grains counted at each interval following the same pattern. Incubation of fresh blood with inosine seems to have decreased the uptake of labeled adenine and after extended storage

### Table 3.—Estimation of Adenine Incorporated by Quantitation of 3H-label Recovered in Wash Solutions

<table>
<thead>
<tr>
<th>Day</th>
<th>Per Cent of Added Tritium Label Recovered in</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Three Initial Supernates</td>
<td>Glutaraldehyde Supernate</td>
</tr>
<tr>
<td>0</td>
<td>52.3</td>
<td>0.07</td>
</tr>
<tr>
<td>(1 hr. incubation without inosine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 at 4°C</td>
<td>86.9</td>
<td>0.1</td>
</tr>
<tr>
<td>14 at 4°C</td>
<td>60.5</td>
<td>0.17</td>
</tr>
<tr>
<td>21 at 4°C</td>
<td>43.5</td>
<td>0.17</td>
</tr>
<tr>
<td>35 at 4°C</td>
<td>33.1</td>
<td>0.3</td>
</tr>
<tr>
<td>35 at 4°C</td>
<td>34.0</td>
<td>0.2</td>
</tr>
<tr>
<td>(1 hr. incubation with inosine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 at 4°C</td>
<td>40.7</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* This includes five PBS washes in addition to three initial supernates and the glutaraldehyde supernate.
the utilization of adenine appeared to be enhanced. Statistical analysis indicates about a one-in-15 likelihood that any of these results were fortuitous. Figure 2 contains photomicrographs of autoradiograms of representative experiments.

Quantitation of the total radioactivity removed by the washing procedure was also performed with the adenine studies. The data summarized in Table 3 in a general way parallel the pattern of change in the total grain counts given in Table 2. The loss of radiolabel from the red blood cells with the addition of glutaraldehyde was less than 0.3 per cent. The PBS washes removed more radioactivity with increase of storage time, but the final PBS wash contained only an average of 0.3 per cent of the total radioisotope added initially.

DISCUSSION

Autoradiography of soluble labeled compounds in tissues requires elaborate techniques to prevent diffusion prior to freezing or fixation, and to avoid leaching and displacement of the soluble radioisotopes by histologic reagents and the conventional methods for preparing autoradiograms.1,2 Diffusible metabolites remain diffusible even after fixation of the cells. The red cell membrane becomes more permeable during drying of a smear and even phosphorylated compounds, which cannot escape from the intact corpuscle, diffuse into the surroundings with great rapidity. Prior "hardening" of the cell membrane by glutaraldehyde appears to retard diffusion during fixation. Rapid drying leaves less time for diffusion to occur. We have performed studies which show that both conditions are necessary. Applying the nuclear emulsion as a predried film is the third essential condition.3 Bifunctional aldehydes such as glutaraldehyde, may also act by crosslinking adenine and other compounds containing amino groups with the amine groups of proteins, forming Schiff bases.4

It is necessary to use relatively large doses of labeled compounds because of the relatively low rate of metabolic activity in red blood cells. The addition of 100 $\mu$Ci. of glucose-6-3H (specific activity 2.3 Ci./mmole) can be expected to result in about 17 disintegrations per corpuscle per 24 hours at a rate of glucose utilization of 2 $\mu$moles per ml. of packed red cells per hour.17-21 This assumes a closed system of metabolism and does not take into account the production of lactic acid and its loss by diffusion from the intact red cell, and also assumes the production of a silver grain for every $\beta$-particle. The data published by Simon, Chapman and Finch22 and Sugita and Simon23 indicate that the net gain in ATP at the end of six weeks storage of blood in ACD solution with added adenine is approximately 1 $\mu$mole/ml. of red blood cells. It was therefore necessary to add 60 $\mu$Ci. of adenine-8-3H per ml. of blood to produce sufficient numbers of grains in the autoradiograms.

The changing rate of metabolism of glucose by red blood cells stored in ACD solution was selected as the model system to test the technique of autoradiography described in this paper. During storage for five weeks in ACD solution the mean percentage of cells containing no grains, indicating a low level of phosphorylation of glucose, rose from 0.5-51.4 per cent (Table 1, Fig. 1). Simultaneously the percentage of cells with large numbers of grains indicating active metabolism of glucose fell to zero. Incubation of the "run-
"Diffusible Compounds in Human Nonnucleated Erythrocytes"

633

down" red cells with adenosine regenerated their ability to metabolize glucose as shown by the return of the autoradiogram to a "super-fresh" state (Table 1, Fig. 1). It may be assumed that the decline in ability to metabolize glucose parallels the decline of ATP concentration during storage and although we did not measure the concentration of the nucleotides, they are known to be regenerated by the action of adenosine. Decline in the activity of hexokinase and other corpuscular enzymes would have the same effect.

It was also noted that there was progressive loss of the biconcavity of the corpuscles and the appearance of crenated spheres during storage as described by Weed. These changes also parallel the decline in ATP concentration. The total number of grains in 1000 red blood cells declined to an average of 10.9 per cent (range 7.0–17.0 per cent) by the end of five weeks of storage in ACD. These data are comparable to the drop in glucose utilization to 21 per cent during storage of ACD blood reported by Simon.

The quality of the autoradiograms obtained with adenine-8-3H was somewhat better probably because of the action of the glutaraldehyde in binding the amine group of adenine to protein amino radicals. Progressive incorporation of adenine into the nucleotide pool during the first five weeks of storage at 4°C is reflected by the progressive increase in the percentage of corpuscles with larger numbers of grains and the increase of the total number of grains in the 5000 cells counted in the five experiments (Table 2, Fig. 2). The decline in total grain count and the rise in percentage of red blood cells with less than four grains per cell after 42 days of storage is not surprising because these samples were the only ones with significant hemolysis and had a high percentage of crenated spherocytes. Furthermore, the number of grains related to any cell is not an accurate cumulative account of the amount of adenine incorporated into its nucleotide pool (ATP, ADP, AMP, IMP) because the red cell 5'-adenylic acid deaminase can convert AMP to IMP by deamination of the adenine moiety. The IMP is then further degraded to hypoxanthine, which diffuses freely through the membrane, and ribose-1-phosphate.

It is interesting that incubation for one hour resulted in as much elevation of intracellular grains as storage for five weeks at 4°C. Beutler has observed regeneration of 2,3-DPG in stored blood following incubation of the red cells at 37°C with fresh plasma. The potential value of incubating red blood cells in their own plasma with adenine added prior to storage or following storage seems worthy of further study.

Inosine is known to be useful mainly for regenerating red cells which have been stored and to be of little utility for maintaining red cells in storage if added at the time of collection. The data in Table 2 suggest that inosine actually suppresses the utilization of adenine when incubated with fresh blood. The autoradiograms did not detect any effect of incubating 10 mM inosine with red blood cells stored for five weeks and the improvement in the total grain count and grain distribution in an identical study after six weeks was at the borderline of significance. However, both the preparations with and without added inosine were incubated for one hour at 37°C to maintain comparable experimental conditions, and it can be seen in Fig. 2d that the ap-
pearance of the erythrocytes was considerably improved in the presence of inosine.

Retardation of the incorporation of adenine by adding inosine to fresh blood may be related to Banaschak’s observation that inosine enhanced the disappearance of ATP from stroma-free hemolysates with the formation of IMP. The competition of this reaction for ATP could result in slowing the formation of adenine nucleotides. The input of trioses formed from the ribose moiety of the inosine into the Embden–Meyerhof pathway would, however, be expected to increase the synthesis of ATP and result in increased formation of nucleotides from added adenine. At the same time slowing of the rate of glucose metabolism can result as observed by Murphy. The overall effect, to a great extent, is dependent on the metabolic state of the red blood cells. When inosine is added to blood with markedly reduced levels of ATP and 2,3-DPG these compounds are regenerated. The regeneration of ATP may occur as the result of the formation of AMP by the reaction of the phosphoribosylpyrophosphate formed from the ribose phosphate split from the added inosine. This step is catalyzed by a specific pyrophosphorylase and the resulting AMP is converted to ADP by the action of adenylate kinase in the presence of ATP. The 2,3-DPG is synthesized in the Embden–Meyerhof pathway from the trioses produced from the ribose phosphate split from the inosine by the action of nucleoside phosphorylase.

When 0.5–0.6 μmoles of adenine are added per milliliter of stored blood, all of it is metabolized by the end of six weeks storage at 4°C. Our data suggest that the incorporation of adenine is slow and cumulative under these conditions until the corpuscular membrane is irreversibly damaged.

Autoradiography of diffusible radioisotopes in mature red corpuscles is useful for studying the metabolic heterogeneity of populations of red blood cells. It can be used for the investigation of hereditary and acquired metabolic abnormalities of erythrocytes. It may provide a method for the in vitro assessment of the potential effectiveness of new techniques of blood preservation. Proof of the prognostic validity of autoradiograms in predicting the survival of populations of red blood cells will rest on in vivo survival studies performed simultaneously on samples of the same blood specimens.

Summary

A simple method for autoradiography of diffusible substances in erythrocytes is described. It is based on retarding diffusion from the red corpuscles during fixation and processing by using the bifunctional aldehyde, glutaraldehyde; rapid drying; and applying the nuclear emulsion as a dried, preformed film.

The technique was used for studying the changes during storage in the ability of red blood cells to utilize glucose. The incorporation of glucose-6-3H by samples of blood stored for different periods of time was followed by the number of silver grains present in the red corpuscles. It was observed that the percentage of erythrocytes with fewer than four grains increased progressively during the storage of ACD blood. The incorporation of glucose as observed by the silver grain pattern was reversible toward that of freshly col-
lected red corpuscles following incubation of the stored samples with adenosine.

Similar studies were performed using adenine-8-1H as the label in blood stored for six weeks in ACD adenine solution. Incubation of the blood samples containing 0.5 mM. adenine for one hour at 37°C resulted in autoradiographic patterns equivalent in grain counts to those given by red blood cells stored with the radiolabeled adenine for five weeks. The incubation of fresh samples with inosine was found to inhibit the incorporation of adenine, whereas inosine enhanced the number of grains in red blood cells which had "run down" during storage.

The autoradiographic technique presented should be of value for the in vitro evaluation of methods of blood preservation and for studying the metabolic heterogeneity of normal red corpuscles and other tissue cells. It should be useful for investigating hereditary and acquired abnormalities of metabolism.

ACKNOWLEDGMENT

The authors thank Mohamed Bakry for performing the liquid scintillation counts.

REFERENCES

17. Murphy, J. R.: Erythrocyte metabo-