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ION-RETARDATION DESALTING OF BLOOD AND OTHER ANIMAL TISSUES
FOR SEPARATION OF SOLUBLE METABOLITIES BY TWO-DIMENSIONAL CHROMATOGRAPHY

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Running Head: Desalting Of Tissue Extracts

Abstract

Metabolites present in acid extracts of mammalian tissues were desalted by passing the extracts through AG11A8 ion-retardation resin. Quantitative recoveries of alanine, aspartate, glucose, glutamate, glutamine, lactate, leucine and maltose were: 95, 100, 92, 85, 96, 90, 97, and 100 percents, respectively. Effective desalting allows metabolites present in tissue extracts to be separated by two-dimensional paper chromatography.

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Kinetic tracer studies utilizing two-dimensional chromatography, autoradiography, and semi-automated Geiger counting as developed by Bassham and Calvin (1, 2) possess enormous potential for the study of metabolic pathways and their regulation. This capability has been demonstrated by significant contributions to the understanding of the path of carbon in photosynthesis (2) and its regulation (1). Recently, these techniques have also been applied successfully to studies on animal cells in culture (3, 4). A logical progression is to utilize two-dimensional chromatography and the other complementary techniques to study the pathways of carbon flow in animals. In the past, however, salt contents of even small aliquots of physiological solutions have interfered with successful development of radiochromatograms, thereby preventing application of this technique to studies of animal metabolism. As several grams of tissue may be needed after isotope infusion in animals to obtain sufficient radioactivity for quantification, the reduction of salt content in animal tissue extracts in order to render them suitable for separation by two-dimensional chromatography is necessary. The purpose of the present project was to develop techniques for the desalting of mammalian blood and other tissue samples for separation of metabolites by two-dimensional chromatography. This paper describes a desalting procedure utilizing AG11A8 ion-retardation resin. Data indicating good recovery of standards from blood, and radiochromatograms demonstrating that desalted extracts give distinctly resolved chromatograms are presented. Data on less successful methanol extraction and ion-exchange electrolysis procedures are also presented.
Methods

Human Blood: 0.01 uCi of $^{14}$C-glucose, lactate and alanine, singularly and in combination, were added to 10 ml samples of human blood. Protein was removed by the addition of two volumes of ice cold 6% HClO$_4$ and centrifugation. The protein precipitate was washed once by resuspension in 5 ml HClO$_4$ and centrifuged. The pooled extract (30 ml) was applied directly to a 2 x 60 cm column of Dow AG1X8 resin and the column was then eluted with water at the flow rate of 1 ml/min. One hundred fractions of 3 ml volume were collected and tested for radioactivity (by liquid scintillation) and for Cl$^-$ content (by AgNO$_3$ in the presence of HNO$_3$). Salt-free fractions containing radioactivity were pooled, freeze dried, and resuspended in 1.0 ml of 10% methanol. Aliquots of this solution were applied to the origins of Whatman No. 1 chromatographic paper. Each chromatogram was first developed for 24 hours in a solvent made up of 800 ml "liquified" phenol (J.T. Baker USP, about 88% phenol, 12% water), 160 ml water, 10 ml glacial acetic acid, and 1 ml 1.0 M ethylenediaminetetra-acetic acid (EDTA). After drying, the paper was turned 90° and run for 20 hours with butanol-water-propionic acid (50:28:22). After chromatograms were dried for the second time, the location of metabolites was detected by autoradiography (12).

After each run of a column the resin was regenerated by washing with two column volumes of a mixture of 0.5 M NH$_4$OH and NH$_4$Cl, two volumes of 1.0 M NH$_4$Cl, and copious amounts of distilled H$_2$O.

Rat Tissues: In experiments on intact rats 15 to 20 uCi of $^{14}$C-glucose or lactate were infused into post-absorbptive animals. One hour after the pulse infusions the rats were killed and decapitated. Blood (1-2 g) was collected in pre-weighed centrifuge tubes containing 10 ml of 6% HClO$_4$. Liver and quadriceps muscle samples (1-2 g) were dissected out, frozen in liquid N$_2$ and homogenized in ice cold HClO$_4$. Acid extracts (3 ml) were added
directly to 1 by 12.5 cm columns (7, 8) and eluted at the rate of 2 ml/min. Alternatively, prior to passage through the column, acid extracts were neutralized by addition of KOH, and the precipitated KHClO₄ was removed by centrifugation. Salt free fractions collected off the column were pooled, reduced in volume by lyophilization, and separated by two-dimensional chromatography.

In other experiments, 1.0 uCi of ¹⁴C-glucose, alanine, aspartate, glutamate, leucine and maltose (ICN, Irvine, CA) were added individually to 6 ml samples of whole blood. Protein was precipitated by the addition of two volumes of cold 6% HClO₄ followed by centrifugation. The acid extracts were then neutralized with KOH and centrifuged. Water was added to give a final dilution of 3:1. Three ml aliquots of these neutralized extracts (equivalent to 1 ml of blood) was added to 1 x 12.5 cm columns of AG1X8 resin and eluted with H₂O at the flow rate of 2 ml/min. After thirty fractions (4 ml each, collected over 2 min) were obtained, 1.0 M NH₄Cl was passed through the column to check for residual activity bound to the resin. Twenty additional fractions were then collected with NH₄Cl as the eluent. Fractions were tested for radioactivity by liquid scintillation counting. To obtain a precise estimate of salt retardation, ²²Na⁺ and ³⁶Cl⁻ were added separately to blood samples and each sample was treated as described above in the case of ¹⁴C-tagged metabolites.

Fractions 1 through 10, representing the first 40 ml off the column, were pooled, lyophilized, and resuspended in 1 ml of 10% methanol and applied to the origins of chromatography paper. Two-dimensional chromatography was performed as noted above.

Methanol Extraction: The procedures described by Hubbard (9) were employed to prepare tissue extracts for paper chromatography. ¹⁴C tracers were added to 10 ml samples of blood, and blood was deproteinized and neutralized as described above. Following neutralization, the extract was freeze dried, and contents of freeze drying vessels were washed 5 times with 5 ml of 80 or 100%
methanol. The combined extracts were dried by evaporation under N₂. This residue was resuspended in methanol and applied to paper for separation by two-dimensional chromatography.

Ion-exchange electrolysis: The procedures of Hubbard were modified to include an additional step for salt removal by ion-exchange electrolysis. This step preceded lyophylization and utilized the Baird-Tatock CD-1 apparatus (Essex, England). Following electrolysis treatment, aliquots of blood extracts were tested for metabolite recovery by liquid scintillation counting and two-dimensional chromatography.

**Results**

**Human Blood:** Desalting of human blood extracts by ion-retardation column chromatography gave acceptable results (Figure 1A). With an acid extract added to the column and a flow rate of 1 ml/min, radioactivity starts to elute from a 2 x 60 cm column at about 80 ml, peaks at approximately 120 ml, and tails off at approximately 180 ml. Cl⁻ is detected shortly thereafter, at approximately 200 ml. Recoveries from the column of glucose, lactate and alanine added to blood are 90% or better. Ion retardation column desalting has allowed preparation of satisfactory autoradiograms from 10 ml samples of human blood.

Attempts at producing radiochromatograms from extracts of human blood by methanol extraction procedures were unsuccessful (Figure 1B). Use of absolute methanol for metabolite extraction was superior to use of 80% methanol, but results (Figure 1B) were inferior to the ion-retardation procedure (Figure 1A).

Desalting of blood by the ion-exchange procedure produced distinctly resolved radiochromatograms. Recoveries of lactate and alanine, however, were only 20% and 30%, respectively. Attempts at recovery of lactate
in the outer electrode compartment of the electrolysis apparatus were unsuccessful, suggesting that lactate was retained in or on the exchange membrane.

Rat Tissues: Fractions of rat tissues desalted by means of ion-retardation column chromatography and subjected to two-dimensional chromatography produced highly resolved radiochromatograms of blood, liver and muscle (Figures 2A, B and C, respectively) sampled in vivo from rats infused with 14C-glucose. Similar results have been obtained on rat blood following lactate infusion. Quantitative recoveries exceed 90% of the activity originally present in perchloric acid extracts. Radiochromatograms of blood indicate that the preponderance of label is in glucose and lactate, with significant incorporations also into alanine and glutamine.

Results of experiments to determine quantitative recoveries of 14C tagged metabolites, 22Na+ and 36Cl−, are given in Table 1 and Figure 3. Clear separation of 14C metabolites and Cl− is achieved. The quantity of chloride ion present in approximately 1 gram of tissue was retarded to the extent that it was not quantitatively recovered to any significant extent until the NH4Cl wash front appeared. As opposed to 36Cl−, 22Na+ elution was bimodal. One peak coincided with the peaks of 14C metabolites, while the other followed the NH4Cl wash. Organic metabolites, therefore, emerge from the column as salts of monovalent cations. Following NH4Cl washing, salt removal from 1 x 12.5 cm columns required washing with 5 liters of distilled H2O to remove residual Cl−.

Discussion

It appears that the technique of ion-retardation column desalting using Dow AG11A8 resin will facilitate radio-tracer studies involving the sampling of blood in vivo. Recoveries of the Glucose-alanine Cycle metabolites and glutamine are good (90+%). Radiochromatograms developed from blood sampled from animals previously infused with 14C tracers in vivo indicate clear
separations of several metabolites into which label had been incorporated. These chromatograms indicate that existing methodologies used in conjunction with glucose and lactate turnover studies (6, 10) may have been inadequate as they failed to consider the various avenues of carbon flow. If one simply observes the specific activities of lactate and glucose following lactate infusion, then one will underestimate the conversion of lactate to glucose as any tracer converted first to alanine, pyruvate, or glutamine, and then to glucose, will bias the calculated lactate to glucose conversion.

In addition to facilitating tracer studies on blood, the ion-retardation resin procedure has similar potential for making possible kinetic tracer studies on other mammalian tissues sampled in vivo. Recovery of label from the column for liver and muscle extracts when glucose was the $^{14}$C source was high (90+%), and the radiochromatograms indicate the existence of several amino acids and phosphorylated compounds.

It is obvious that the study of complex metabolic processes within an intact animal, where recycling and recirculation of label occur, requires the use of computer models (13, 14). It is equally certain, however, that validity of these models depends upon identification and quantification of pool sizes and specific activities of metabolites as a function of time. When used in conjunction with single, dual, and specific labeling techniques, the ability to desalt tissue extracts for separation of metabolites by two-dimensional chromatography becomes important.

The procedures to desalt, separate, and quantify metabolites present in extracts of mammalian tissues may allow a new, dynamic approach to the study of metabolism in vivo. The initiation of kinetic tracer studies on intact animals and their respective tissues (blood, liver, spleen, kidney, heart, brain, red and white skeletal muscle) under various physiological conditions (physical
exercise, dietary deficiency, starvation, environmental stress, and disease), would complement the types of studies previously possible (5, 6, 9, 10, 11).
References


Acknowledgements

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Legends

1. Figure 1: Two-dimensional radiochromatograms developed from 10 ml of human blood to which 0.01 uCi of $^{14}$C glucose, lactate and alanine were added. Radiochromatogram (A), top, was developed from blood subjected to the present ion-retardation procedure. Radiochromatogram (B), bottom, was developed using a methanol extraction procedure (9). Note improved resolution in (A) compared to (B). Two-dimensional chromatography and autoradiography were performed as described previously by Bassham, Calvin, and associates (12).

2. Figure 2: Two-dimensional radiochromatograms of rat tissues sampled in vivo one hour following infusion of 20 uCi of $^{14}$C glucose into a resting, post-absorptive rat, (A) blood, (B) liver, and (C) muscle.

3. Figure 3: Fractionation of tracers present in neutralized extracts of blood eluted from a 1 x 12.5 cm column of AG11A8 ion-retardation resin. Flow was 2 ml/min.
Table 1: Recoveries of tagged species present in neutralized rat blood extracts added to a 1 x 12.5 cm column of AG11A8 resin.+

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak (ml)</th>
<th>% Recovery @ 40 ml</th>
<th>% Recovery @ 60 ml</th>
<th>% Recovery ++ in 1.0 M NH₄Cl</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>¹⁴C Alanine</td>
<td>16</td>
<td>94.77</td>
<td>96.02</td>
<td>0.91</td>
<td>4</td>
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<tr>
<td>¹⁴C L-ascorbic acid</td>
<td>15</td>
<td>105.44</td>
<td>106.45</td>
<td>2.09</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Glucose</td>
<td>16</td>
<td>91.87</td>
<td>92.78</td>
<td>0.73</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Glutamate</td>
<td>16</td>
<td>84.70</td>
<td>85.92</td>
<td>9.12</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Glutamine</td>
<td>16</td>
<td>95.83</td>
<td>98.70</td>
<td>1.25</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Lactate</td>
<td>20</td>
<td>89.61</td>
<td>95.12</td>
<td>1.68</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Leucine</td>
<td>16</td>
<td>96.91</td>
<td>97.69</td>
<td>0.80</td>
<td>4</td>
</tr>
<tr>
<td>¹⁴C Maltose</td>
<td>12</td>
<td>99.68</td>
<td>99.80</td>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>³⁶Cl⁻</td>
<td>140</td>
<td>0.34</td>
<td>2.471</td>
<td>86.60</td>
<td>7</td>
</tr>
<tr>
<td>²²Na⁺</td>
<td>16; 140</td>
<td>35.54</td>
<td>45.63</td>
<td>31.37</td>
<td>6</td>
</tr>
</tbody>
</table>

+Flow = 2 ml/min

Tracers added to the column in the form of neutralized, acid extracts of blood; dilution = 3:1.

Volume added to column = 3 ml

++Thirty fractions (4 ml each) collected with distilled water as the eluting medium. This was followed by 20 fractions with 1.0 M NH₄Cl as the eluent.
Ion retardation desalting
(Human Blood)

(A)

Phenol - water - acetic acid, 24 h

Lactate
Alanine
Glucose

(B)

Methanol wash desalting
(Human Blood)

Phenol - water - acetic acid, 24 h

Lactate
Alanine
Glucose

Fig.
Fig. 2
Fig. 3
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