Quantitative Studies on the Influence of the Temperature Applied in Freeze-Substitution on P, N, and Dry Mass Losses in Fixed Tissues

Different laboratories apply various temperatures in the freeze-substitution (F-S) technique, an attempt was, therefore, undertaken to study the influence of the temperatures applied on the extractability of certain substances and on the loss of dry tissue mass. The choice of the substituting medium and of temperature is well known [1-3] to be of essential importance for adequate fixing of tissues. In a preceding paper [4] dealing with the appropriateness of various media for the F-S technique, it was established that tissue substituted in acetone shows no loss in dry mass, N and P, even if the substitution lasted many days. Methanol, on the contrary, though frequently used as substituting medium extracted in the course of substitution a large part of N and P containing compounds. The aim of the present paper was to establish the influence of acetone and methanol temperature on the eventual losses in dry mass, N and P in tissues substituted with these substances. This matter is of no small interest because the temperature of the substituting medium influences the rate of its permeation into the tissues and consequently the substitution rate.

Material and Methods. As experimental material the livers of 5 female rats of the Wistar strain, weighing ca. 150 g each, were used. After weighing, the material was cooled in isopentane at −175°C and placed in 5 ml portions of the substituting medium. From each liver 6 samples were taken plus 3 controls. The material was substituted in acetone or methanol at −79°, −20°, and +4°C, respectively for 3 days. Afterwards it was dried to constant weight at 100°C in order to determine the percentage of dry mass and compare it with the control. N and P were determined by the methods described elsewhere [4]. The quantity of P extracted into the medium was also determined and a balance of the amount of tissue P and P recovered from the medium was set up. Statistical calculations were made.

Results. The results are presented in the Table. P in the medium was only determined for tissues substituted with methanol, because the P loss in acetone was negligible. The sum of P found in the tissues and in methanol was close to 100% within the limits of methodic error.

Discussion. The discussion concerning the quality of the substances extracted into the substituting medium has been given in the preceding paper [4]. In the experiments described above the following temperatures were applied: −79° (mixture of dry ice with methanol), −20° (deep-freeze refrigerator) and +4° (refrigerator temperature). The frequently applied temperature of −40° has been omitted, since it could be anticipated that mean results would be obtained. It was found that the losses in dry mass N and P are considerably smaller in substitution with acetone than with methanol. As may be seen from the data in the Table, the changes in the percentage of dry mass caused by its extraction into methanol depend on the temperature applied. A statistically significant difference was noted not only between the substituted material and the control but also between tissues substituted at −79° and at +4°C. Tissue N losses after substitution with methanol were statistically significant only in comparison to the control samples, whereas no effect of the temperature applied could be demonstrated. The greatest changes were observed in the extraction of P-containing compounds with methanol. At −20° and +4°C they were of the same order (ca. 60%), whereas at −79° the loss was considerably smaller (ca. 40%). It should be stressed that the time of substitution (much shorter than in the preceding work [4]) does not, in principle, influence the percentage of P loss. The superiority of acetone as substituting medium is, in the light of these experiments, worth stressing.

Conclusions. (1) The influence of temperature applied in the course of F-S on the extractability of N and P, as well as on the loss of dry mass when using methanol, has been demonstrated. (2) In view of the low P, N and dry mass losses confirming the results of earlier studies—in tissues substituted with acetone—it was not possible to establish any relation between the temperature applied and these

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The content of N, P, and dry mass of rat liver after the substitution in methanol and acetone in the different temperatures. Results expressed as %-values of the control material

	Control			Methanol −79°C			−20°C			+4°C		
	% of dry mass	mgN/100 mg[a]	μgP/100 mg[a]	% of dry mass	% of content N	% of content P	% of dry mass	% of content N	% of content P	% of dry mass	% of content N	% of content P
Mean	29.5	3.66	529.0	25.9	96.7	60.6	24.3	96.5	46.1	24.2	95.7	44.8
Standard deviation	±0.4	±0.07	±37.0	±0.8	±0.7	±3.3	±0.9	±1.6	±2.7	±0.4	±1.3	±1.8
Variation coefficient	1.4	1.9	7.0	3.1	0.7	5.4	3.5	1.7	5.9	1.6	1.1	4.0
				Acetone								
Mean				30.9	100.9	97.5	31.0	101.2	98.5	30.7	100.3	94.1
Standard deviation				±1.4	±2.5	±2.0	±1.4	±2.4	±3.6	±1.0	±2.5	±0.7
Variation coefficient				4.5	2.5	2.1	4.4	2.3	3.7	3.3	2.5	0.7

[a] of fresh weight

losses. (3) Application of low temperature (– 79°C) in the course of substitution seems to be justified.

Résumé. Les auteurs ont constaté que la température a une influence sur la diminution du contenu en azote et en phosphore et sur celle de la masse déséchée après l'usage de méthanol au cours de la congelation-dissolution (freeze-substitution) des tissus. Ces diminutions sont moins marquées à la température de – 79°C. Après l'usage d'acétone, les diminutions atteignent leur minimum.

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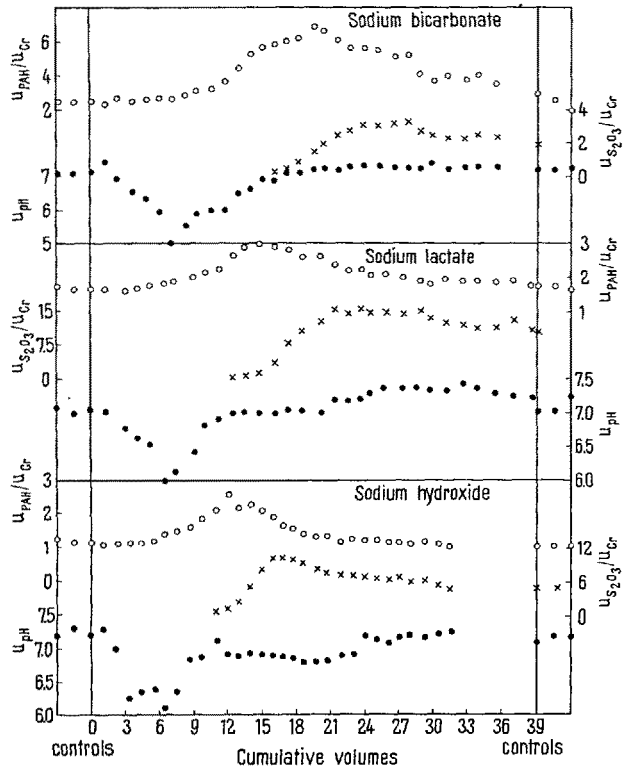
Stop-Flow Studies of Alkaline Urines

When an alkaline urine is produced, it has been found that the pCO_2 of the final urine may exceed that of the arterial blood by a considerable margin[1,2]. To explain this phenomenon, PITTS and LOTSPEICH[1] suggested that during formation of an alkaline urine, the distal tubule continued to secrete hydrogen ions in exchange for sodium ions, that such secretion led to the conversion of HCO_3^- to H_2CO_3, and that the delayed conversion of H_2CO_3 to CO_2 and water leads to a high urinary pCO_2. The authors are aware of no experiments designed to test this hypothesis directly. One of its predictable consequences is that acidification occurs in a distal segment despite the production of a final alkaline urine. This paper reports that such acidification can be demonstrated by means of the stop-flow technique[3].

Male 15 kg dogs were anesthetized with nembutal. The ureters were exposed, and catheters were inserted to the pelvis, and the free ends of the catheters brought out through flank incisions in preparation for carrying out stop-flow experiments to localize sites of pH change. In some dogs, either isosmotic $NaHCO_3$ or Na lactate solution containing PAH was infused into one femoral vein at a rate fast enough (3–6 ml/min) to cause production of a frankly alkaline urine. The remaining animals to be considered in this study were infused with NaOH by way of a catheter inserted into the jugular vein, and forced deeply enough so that its opening was at the expected level of the superior vena cava. After the urine had become adequately alkaline, infusion of 20% mannitol without any added salt was begun while continuing infusion of the alkalinizing solution. Sodium chloride was omitted since it was found that the urine pH would drop to the acid range if the mannitol solution contained salt. When urine flows became greater than 6 ml/min, a 6 min stop-flow experiment was run. The urine samples were collected manually and immediately covered with mineral oil to minimize pH changes. Because of the ease of analysis, S_2O_3 was used as a glomerular marker and injected 30 sec before the end of uretral clamping. PAH was used as a proximal marker.

The Figure shows typical results of experiments carried out by each of the methods utilized for inducing urinary alkalinization. In all experiments it was found that the pH of the urine coming from distal sites became acidified, even though the free flow urines before diureses never had a pH lower than 7.6 and ranged up to 8.5. The Table summarizes pertinent data on all experiments. No correlation was indicated between the distal pH drop, and free flow urinary pH or nature of the alkalinizing salt. No clear cut evidence for the locus of alkalinization was obtained.

It is assumed that the establishment of concentration gradients under stop-flow conditions are reflections of processes which occur in the elaboration of urine under free flow conditions. If such is indeed the case, then it may



Graphical representation of single experiments where alkalinization was produced by infusion of the salt indicated. Proximal regions of the nephron population are indicated by the peaked portion of the PAH curve. New glomerular filtrate is indicated by the rise in urinary S_2O_3 concentration. In all sections of the Figure the open circles show the PAH ratios, the crosses the S_2O_3 ratios, and the closed circles the urinary pH.

Magnitude of distal pH change in dogs loaded with an urinary alkalinizing salt

Salt	No. of experiments	Urinary pH[a] no diuresis	Urinary pH diuresis	Change distal pH
$NaHCO_3$	3	7.6–8.5	7.1–7.5	0.9–2.3
Na lactate	4	7.7–8.1	7.2–7.6	0.9–1.2
NaOH	4	7.9–8.2	7.0–7.4	0.8–1.3

[a] Numbers show ranges of the data.

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