Quantitative Investigations on the Solubility of Proteins Extracted from Tissues Fixed by Different Chemical and Physical Methods

The development of quantitative histochemistry caused a series of investigations concerning the possible loss of particular substances from the examined tissues during histological procedure, knowledge of the extent of such losses in case of different substances, tissues and techniques is necessary to choose the best suited procedure in each instance, as well as for the critical evaluation of the results obtained.

The aim of this work was to investigate the possible losses of proteins from tissues following different methods of fixation. Only the influence of fixation was investigated, the changes caused by the remaining steps of histological procedure being omitted from our considerations.

Similar investigations were already carried out, with exception of 'freeze-substitution' technique. The results are, however, contradictory or non-comparable because of differences in histological procedure or chemical determinations of proteins and/or nucleic acids[1–17].

Material and methods. The liver and kidneys of white rats were used throughout this investigation. Immediately following the sacrifice of the animals, the fragments of tissue standardised in dimensions of 85–220 mg ± 1 mg weight were fixed by one of the following methods: 1. absolute alcohol—24 h. 2. acetone—24 h. 3. 10% formalin—24 h. 4. Carnoy's fluid—24 h. 5. freeze-substitution by butanol cooled to —40°—48 h. 6. freezing-drying in —40° —10 h. The weight of tissues was controlled to determine the degree of drying. 7. unfixed tissues were used as controls.

Each tissue sample was shaken out with 5 ml of distilled water, following chemical or physical fixation, during 2 h. The fluid was filtered and its nitrogen content was determined, using micro-Kjehldahl's method according to PARNAS and WAGNER[18]. The sensitivity of this method permits the determination of 0.01 mg of nitrogen.

Tab. I. Total N and protein content of water extracts of liver fixed by different methods

Method of fixation	Number of experiments	Total N		Extraprotein N		Proteins	
		Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Controls unfixed	10	1.02	0.18	0.06	0.04	6.04	1.36
Absolute alcohol	10	0.34	0.06	0.05	0.03	1.78	0.79
Acetone	10	0.49	0.06	0.04	0.05	2.82	0.09
10% formalin	10	0.00	0.00	0.00	0.00	0.00	0.00
Carnoy's fluid	10	0.05	0.02	0.00	0.00	0.30	0.15
Freeze-substitution	10	1.15	0.17	0.07	0.03	6.78	1.23
Freezing-drying	9	1.30	0.41	0.06	0.04	8.15	2.54

Results expressed as mg of nitrogen or proteins on 100 mg fresh weight of rat liver.

Tab. II. Total N and protein content of water extracts of kidneys fixed by different methods

Method of fixation	Number of experiments	Total N		Extraprotein N		Proteins	
		Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
Controls unfixed	10	1.11	0.27	0.13	0.03	6.14	1.86
Absolute alcohol	10	0.34	0.06	–	–	–	–
Acetone	10	0.79	0.13	–	–	–	–
10% formalin	10	0.00	0.00	0.00	0.00	0.00	0.00
Carnoy's fluid	10	0.05	0.01	0.00	0.00	0.30	0.08
Freeze-substitution	10	1.22	0.32	0.13	0.03	6.86	2.19
Freezing-drying	10	1.33	0.32	0.13	0.03	7.53	2.19

Results expressed as mg of nitrogen or proteins on 100 mg fresh weight of rat kidneys.

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To determine the extra-proteic nitrogen, a similar series of experiments was carried out with the difference that the proteins were removed from the fluid by phenol (KIRBY[19]).

Results. The results obtained are presented in Table I for liver and Table II for kidneys.

The nitrogen content was determined also in the remaining fixatives, i.e. in formalin and alcohol. On the average, formalin contains 0.13 mg of N for 100 mg of liver tissue, alcohol –0.20 mg of N for 100 mg of liver tissue.

The method applied in the present work does not permit us to determine the content of DNA-nitrogen, because this remains in the phenol fraction. DNA elued does not exceed 1% of fresh tissue weight, so that authors feel their assumption that total extra-proteic nitrogen was determined to be justified.

Conclusions. 1. The quantity of proteins elued from lyophilized material and controls is equal, differences noted being statistically insignificant. 2. The method of freeze-substitution is comparable as regards protein elution to lyophilization and controls. 3. The elution of proteins following chemical fixation, i.e. in formalin and Caroy's fluid, is negligible, being lower than 1% of fresh tissue weight. The quantity of proteins elued following absolute alcohol or acetone fixation may reach 2–3% of fresh tissue weight. Best reproducible results were obtained following acetone fixation. 4. No significant differences were found to exist between liver and kidney tissue.

Résumé. Les auteurs ont examiné l'influence de la fixation sur la solubilité des protéines. Le rein et le foie de rats blancs ont été fixés dans l'éthanol absolu, la formaline et l'acétone par lyophilisation et par la méthode de «freeze-substitution». Les protéines furent extraites par l'eau distillée et leur quantité déterminée par la microméthode de KJELDAHL. Un tableau résume les résultats obtenus.

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Histochemical Analysis of Three Dehydrogenase Systems in the Renal Adenocarcinoma of the Frog *Rana pipiens*

Recently we studied three dehydrogenase systems in the renal adenocarcinoma of the oviparous Toothcarp *Aplocheilus lineatus*[1]. To establish whether a more general significance should be attached to the results obtained, it seemed desirable to us to perform a corresponding investigation with another lower vertebrates. As experimental material, we chose the renal adenocarcinoma in the frog *Rana pipiens*[2], of which 18 males and 26 females were available. Histologically, the tumours, involving one or both kidneys, consisted of atypical cells usually much larger and basophilic than normal kidney cells and of a scanty, poorly vascular stroma. The epithelial tumour cells which can form irregular acinar or tubular structures, sometimes show intranuclear inclusions because the chromatin is condensed about the periphery of the nucleus and is separated from the large central acidophilic inclusion by a clean space. No capsule is present and, even in very small tumours, marginal extensions of tumour tissue infiltrate the surrounding tissues. Mitoses are frequently found. Metastasis is not uncommon, especially in the lungs and liver.

This tumour was studied histochemically for the three dehydrogenase systems, namely succinic diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) diaphorases and the DPN-dependent enzymes: lactic, malic, glutamic, β-hydroxybutyric, and ethanolic dehydrogenases[1,3–6]. To demonstrate mitochondria some sections were also processed with Regaud's stain[7]. In general, for morphological orientation, parallel sections were stained with haematoxylin and eosin or thionin.

As in *Aplocheilus lineatus*[1], also in the tumour tissue of *Rana pipiens* the succinic dehydrogenase activity was low as compared to normal tissues. This enzyme appeared to be linked to the mitochondria and cell membranes. Tumour cells undergoing necrotic changes were inactive or very nearly so, but the intraluminal material revealed a marked enzymatic activity, which was due either to persistent organelles of disintegrating desquamated tumour cells or, which is less likely, to the presence of the enzyme system in the tumour cell secretion.

The DPN diaphorase activity was high, however, similar to that in the most intensely stained normal cells. The DPN diaphorase and the DPN-linked dehydrogenases appeared to be linked to the mitochondria and some cell membranes. The TPN diaphorase activity was observed to be much lower than the DPN diaphorase activity showing a more finely and widely dispersed intracytoplasmic distribution, which is suggestive of the presence of microsomes, and displayed a high concentration in the cell membranes. The glutamic and particularly the ethanolic dehydrogenase activities were only weak in the tumour

Distribution of succinic dehydrogenase, DPN and TPN diaphorases and several DPN-linked dehydrogenases in the renal adenocarcinoma of the frog *Rana pipiens*. The enzymatic activity was rated histologically on the basis of colour reaction as 0 to 4 by inspection of the tumour cells.

Succinic dehydrogenase	1
DPN diaphorase	4
TPN diaphorase	2
Lactic dehydrogenase[a]	4
Malic dehydrogenase[a]	2
Glutamic dehydrogenase[a]	1
β-hydrobutyric dehydrogenase[a]	2
Ethanolic dehydrogenase[a]	0

[a] DPN-linked dehydrogenases

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