ANALYSIS OF SOME METHODS OF DETERMINING FREE RIBONUCLEOTIDES IN ANIMAL TISSUES

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The author concludes from his analysis of the literature that the most probable cause of failure during quantitative estimation of free ribonucleotides is breakdown of nucleoside-triphosphates during removal of the tissue and extraction of the nucleotides. The slightest pain inflicted on the animals causes redistribution of nucleotides in the liver of rats. It is suggested that tissue for investigation be taken from anesthetized animals and frozen immediately in liquid nitrogen. The nucleotides should be extracted at the lowest possible temperatures.

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More than ten years have elapsed since the basic principles of isolation and fractionation of nucleotides were worked out, yet there is still an urgent need for more rapid and effective methods for studying them [8]. A critical examination of progress in this field must therefore prove useful.

The use of the method of gradient elution for fractionating the acid-soluble extract on an ion-exchange resin column revealed the presence of phosphate esters of all ribonucleotides in animal tissues. Neutralized tissue extracts without any preliminary purification were used for analysis [12, 16]. Nevertheless, in the overwhelming majority of subsequent investigations, preliminary purification of the nucleotides was carried out by adsorption on charcoal or precipitation as barium or mercury salts [1-5, 6, 7]. It was found, however, that the preparative losses of material by this method may reach 50% or more [3, 5, 6, 7] in association with definite redistribution of the components in accordance with their degree of phosphorylation because of hydrolysis of nucleoside-triphosphates [3, 7]. It was also found that nucleotides differ in their degree of adsorption on and elution from charcoal, so that their relative concentration in the eluates of nucleotides under a vacuum likewise may probably cause degradation of nucleoside-triphosphates as a result of transfer of the terminal phosphate of ATP to alcohol with the formation of ADP and monoethyl phosphate [10]. Clearly it is only through imperfection of the methods of extraction and fractionation of nucleotides that can account for the fact that, according to different workers, the ATP content in the liver may differ by more than 20 times. As a rule, with a relatively low content of triphosphates, immeasurably higher concentrations of mono- and diphosphates are observed [2, 3, 7, ect.]. Meanwhile, the ratio between nucleoside-triphosphates and nucleoside-monophosphates is considered to be one of the most important criteria for qualitative assessment of the method of nucleotide determination [8, 14]. For these reasons, it was decided to seek improved methods of extracting nucleotides.

By extracting the tissue with 10% trichloroacetic acid in acetone at low temperatures (down to −78°) a product was obtained with a much higher concentration of nucleoside triphosphates than of the corresponding mono- and diphosphates [8, 14]. An extremely important factor here was found to be the speed of removal of the chest tissue and of its immersion in liquid nitrogen. The ATP concentration in the liver frozen 15 sec after removal is 25% lower than that in the liver frozen immediately, and in the case of a delay of 30-60 sec before freezing the ATP content falls by 50% [15]. These changes took place despite effective cooling of the tissue [11], and deamination of purine derivatives was observed even at −13° [9]. Indications of a direct relationship between the ATP content in a tissue and the speed of its immersion in liquid nitrogen are also given by other investigators [13]. It is now clear that even the method used to sacrifice the experimental animals has a significant effect on the absolute content and relative proportion of free nucleotides in the tissue. A higher ATP/AMP ratio was found in the brain of rats sacrificed by immersion of the head in liquid nitrogen, whereas better results were obtained in guinea pigs by decapitation of the animals [8]. Furthermore, a significant decrease in the relative ATP concentration in the liver of rats was observed

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TABLE 1. Concentration of Adenosinephosphates in Liver of Rats during Nociceptive Stimulation (in μmoles/g fresh tissue), $M \pm \sigma$

Conditions	No. of rats	ATP	ADP	AMP	Total
Control	8	2,985±0,097	0,867±0,029	1,102±0,043	4,954±0,130
Nociceptive stimulation	13	2,934±0,098 $t=0.4$	1,453±0,078 $t=5.5$	1,618±0,050 $t=7,0$	6,005±0,226 $t=4,0$

even in cases of a marked excitation response during ether anesthesia. These observations were subsequently confirmed in experiments with weak nociceptive stimulation of rats (by squeezing the tip of the tail with forceps) during induction of anesthesia (Table 1). The decrease in ATP content was relative because of an increase in the absolute content of AMP and ADP. It must be assumed that this effect is more marked during decapitation of unanesthetized animals. The same conclusion is drawn by other workers who observed a marked increase in the content of xanthine and hypoxanthine in the liver of rats decapitated without preliminary anesthesia [9].

The data described above show conclusively that widely used methods of extraction and purification of nucleotides are not quantitative. A clear understanding of the available data must therefore help to avoid the commonest errors.

As a result of my own observations and analysis of information in the literature I suggest that the following general principles be adopted. The tissue for investigation should be obtained where possible from living, anesthetized animals. All procedures possibly stimulating or exciting the experimental animals before anesthesia should be avoided. The tissue should be immersed in liquid nitrogen immediately after removal. All procedures connected with preliminary purification of nucleotides must be withdrawn from the method, including purification by adsorption of charcoal or by precipitation as barium salts. The tissue should be extracted with 10% trichloroacetic acid in acetone at low temperatures, improving the conditions for extraction of the nucleotides and avoiding significant losses of nucloside-triphosphates.

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