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The distribution of inorganic phosphate in a biphasic system consisting of chloroform–methanol–water

The most usual method for the extraction of lipids is the procedure of FOLCH–PI *et al.*[1], by which the tissue is extracted in 20 vol. of chloroform – methanol (2:1), and the extract washed with 0.2 vol. of water or salt solution. Most substances other than the fat-soluble are eluted from the chloroform phase by this washing. The inorganic phosphate (P_i) is of most interest, since a large amount of phosphatide research is done with ^{32}P. Therefore one of the most important things to do in such experiments is to free the lipids from P_i completely. The aim of the work described below is to show how the distribution of P_i between the chloroform phase and the water–methanol phase depends on different factors.

12 ml chloroform are mixed with 6 ml methanol for 5 min, together with 4.5 ml water containing 10 μC $^{32}P_i$ per ml (wash water). After separating into a chloroform phase (13.4 ml) and a water–methanol phase (8.6 ml), the radioactivity in both phases was measured with a flow counter. The actual amount of P_i in both phases was calculated from these values and the specific activity of the P_i. The experiments were performed at a temperature of 30° throughout.

The P_i concentration of the chloroform phase rises linearly with the P_i concentration of the wash water (Table I). The P_i of the chloroform phase is about 0.015 % of the P_i of the wash water with a pH of 4.8 independent of the actual amount of P_i present. This behaviour of the P_i is for example important in experiments where phosphate buffers of high concentration are used. It may then be possible that P_i appears in the washed chloroform phase in amounts which are not only detectable as ^{32}P, but also with the usual methods of P_i determination. These amounts of P_i will interfere with P_{lipid} especially if the amount of lipid is small compared with the volume of the chloroform–methanol extract, which is the case in tissues poor in lipids. Here it is absolutely necessary to wash the extract at least three times.

The influence of pH on the distribution of P_i is shown in Table II. The wash water contained 150 mmoles P_i per ml, which is a total of 675 μmoles P_i, of different pH values. The highest P_i concentration in the chloroform phase is obtained with the lowest pH values, and decreases with rising pH asymptotically. When the P_i concentrations are plotted on a log–log graph, a straight line, decreasing in value, is obtained. These results show that if a lipid extract is washed with 0.1 N HCl, with a pH of 1, as is

TABLE I

THE INFLUENCE OF THE P_i CONCENTRATION OF THE WASH WATER ON THE P_i CONCENTRATION OF THE CHLOROFORM PHASE

Chloroform, 12 ml; methanol, 6 ml; wash water (pH 4.8), 4.5 ml.

Wash water (μmoles) KH_2PO_4	*P_i in chloroform phase*	
	μmoles	*In % of wash water*
$13.5 \cdot 10^{-5}$	$18.5 \cdot 10^{-9}$	0.0137
$4.5 \cdot 10^{-3}$	$8.0 \cdot 10^{-7}$	0.0178
4.5	$5.6 \cdot 10^{-4}$	0.0125
675.0	$1.2 \cdot 10^{-1}$	0.0178

common in many laboratories, the P_i in the chloroform phase is 60 times as high as if it were washed with neutral pH. This finding becomes even more significant with another set of experiments. Chloroform – methanol – water was mixed in the same ratio as is obtained if tissue is extracted with 20 vol. of chloroform – methanol (2:1), *viz.* 16:8:1. This crude extract was saturated with P_i (0.075 mM at pH 6.8) and washed with 0.2 vol. of wash water of different compositions. The crude extract washed with 0.1 N HCl contained still, after washing, $6.5 \cdot 10^{-3}$ μmoles P_i, which only disappeared after the second washing. The chloroform phase contained no detectable amounts of P_i after washing with KCl, NaCl or NaOH. LEBARON AND LEES[2] found a similar detrimental effect of acid pH values, for protein was still dissolved in the washed chloroform extract if the tissue was extracted with 10% trichloroacetic acid prior to the chloroform–methanol extraction.

The percentage of the methanol volume against the chloroform–water volume has a very marked effect on the distribution of P_i (Table III). The P_i concentration

TABLE II

THE INFLUENCE OF THE pH OF THE WASH WATER ON THE P_i CONCENTRATION OF THE CHLOROFORM PHASE

Chloroform, 12 ml, methanol, 6 ml; wash water (150 mM P_i), 4.5 ml., Temp. 30°.

Wash water		*P_i in chloroform phase*	
P_i	*pH*	*μmoles*	*In % of wash water*
H_3PO_4	1.3	2.45	0.363
H_3PO_4/KH_2PO_4	2.0	1.56	0.231
KH_2PO_4	4.8	$1.2 \cdot 10^{-1}$	0.0178
KH_2PO_4/K_2HPO_4	7.0	$4 \cdot 10^{-2}$	0.0059
K_2HPO_4	9.4	$2.4 \cdot 10^{-2}$	0.0036
K_2HPO_4/K_3PO_4	11.4	$1.5 \cdot 10^{-2}$	0.0022
K_3PO_4	12.0	$1.1 \cdot 10^{-2}$	0.0016

TABLE III

THE INFLUENCE OF THE AMOUNT OF METHANOL ON THE P_i CONCENTRATION OF THE CHLOROFORM PHASE

Chloroform, 12 ml; wash water (150 mM sodium potassium phosphate, pH 5.4), 4.5 ml., Temp. 30°

Methanol (ml)	*Water–methanol phase*		*Chloroform phase*		
				P_i	
	ml	*μmoles P_i*	*ml*	*μmoles*	*In % of wash water*
0	4.4	675	12.0	—	—
1.5	5.4	675	12.1	$8.1 \cdot 10^{-4}$	$1.2 \cdot 10^{-4}$
3.0	6.7	675	12.4	$5.8 \cdot 10^{-3}$	$8.6 \cdot 10^{-4}$
4.5	7.7	675	12.8	$1.5 \cdot 10^{-2}$	$2.2 \cdot 10^{-3}$
6.0	8.6	675	13.4	$9.4 \cdot 10^{-2}$	$1.4 \cdot 10^{-2}$
7.5	9.2	674	14.2	$7.3 \cdot 10^{-1}$	$1.1 \cdot 10^{-1}$
9.0	9.6	670	15.1	3.5	$5.2 \cdot 10^{-1}$
10.5	10.1	380	16.1	13.4	2.0
12.0	7.3	257	20.5	97.6	14.5

of the chloroform phase rises progressively with increasing methanol, while a rise of the methanol volume of about 3.6% of the chloroform–water volume causes a doubling of the P_i concentration. If the methanol rises above 50%, an increasing amount of P_i precipitates out of the biphasic system. A change from the usual relationship chloroform–methanol–water (8:4:3), therefore, causes a simultaneous change in the distribution of P_i between the two phases. Such a change appears if the tissue is extracted hot, where chloroform evaporates more than methanol, and therefore the percentage of methanol rises.

The temperature has no influence on the distribution of P_i between the two phases. The results are the same if the experiments are carried out at 2°, 20°, or 30°.

The experiments described show that the cleaning of P_i from the lipids depends very much on the procedure used. The cleaning is better the lower the P_i in the extracted material, the higher the pH of the wash water, and the lower the percentage of methanol in the total mixture. The P_i dissolved in the chloroform phase will not alter the results if the following points are observed: (1) The P_i buffer of the incubation medium should not exceed 50 mM; (2) the specific activity of the P_i should not exceed 1 mC/μmole P_i; (3) the lipid concentration in the chloroform–methanol extract should not be below 1 μmole/10 ml; (4) the crude extract should be washed with neutral pH; (5) the amount of methanol should not exceed 50% that of the chloroform.

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