Experimental condition		PEA µg/l urine mean[a] ± S.E.
a)	Urine extracted without acid hydrolysis (free PEA)	292 ± 45
	Urine extracted after acid hydrolysis (conjugated PEA)	276 ± 62
b)	Urine extracted after acid hydrolysis (free + conjugated PEA)	551 ± 78
c)	Urine extracted after incubation with glucuronidase (free PEA + PEA glucuronide)	515 ± 69

[a] Average of 5 replicates.

reported using the PEA-alloxan fluorometric technique[6] (mean 47 µg/24 h, range 5–167). Values for urinary conjugated PEA levels are not available in the literature.

Recovery studies indicated that over 95% of the free PEA was removed from the urine after a single n-hexane extraction; a second extraction yielded less than 5% more free PEA. However, after the extraction and removal of free PEA from the urine, and the conjugated substances alone were subjected to either acid hydrolysis or incubated with β-glucuronidase under the conditions previously described, additional quantities of PEA were extractable in each case which accounted for approximately 50% to 60% of the total PEA (free plus conjugated). In most urine samples, the PEA values obtained by acid hydrolysis of the conjugated substances were similar to the values obtained by enzymatic hydrolysis. However, in some urine samples, significantly higher values of conjugated PEA were obtained by acid hydrolysis [69.8 ± 15.7 Mean (of 5 replicates) ± S.E.] than by β-glucuronidase degradation [36.0 ± 9.0 Mean (of 5 replicates) ± S.E.]. These results led us to suggest that PEA glucuronide is one of the major excretion products of conjugated PEA, but there may be other forms, probably ethereal sulfates or PEA associated with uric acid.

Zusammenfassung. Nachweis, dass die Ausscheidung von 2-Phenylaethylamin-Glucuronid im Urin von gesunden Versuchspersonen zwischen 50 und 60% der Gesamtmenge beträgt.

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On the Fractionation of Halophilic Enzymes with Ammonium Sulphate

The enzymes from extremely halophilic bacteria require high salt concentrations for both activity and stability[1,2]. The salt requirement for stability makes purification of these enzymes a difficult task, since very few methods can be applied successfully in the presence of NaCl concentrations ranging from 3 to 5 *M*. Ammonium sulphate fractionation, perhaps the commonest procedure in enzyme purification, is very difficult in the presence of high concentrations of monovalent cations[3]. Different ways of avoiding this difficulty have been reported in the literature, namely precipitation on the enzme after removal of the NaCl, followed by renaturation by dialysis against 5 *M* NaCl[3]; precipitation after removal of the salt, protecting the enzyme under study with substrates[4,5], and direct precipitation with solid ammonium sulphate starting with a crude extract in 2 *M* $(NH_4)_2SO_4$[6]. The first procedure usually gives low yields[3,4]; the second can be applied only to enzymes susceptible of protection by substrates or cofactors. The results presented here show that considerable purification, with reasonably good yields, of several halophilic enzymes, can easily be attained by direct fractionation of a crude extract in 5 *M* NaCl with a saturated solution of ammonium sulphate.

The strain of *Halobacterium cutirubrum*, the conditions of growth, harvesting and washing of the cells, the preparation of the cell-free extracts, the DNAase treatment, and the determinations of protein, were as previously described[7,8]. The crude extract, in 0.05 *M* *Tris*-HCl buffer (pH 7.6), containing 5 *M* NaCl and 1 m*M* EDTA, was brought successively to the following concentrations of ammonium sulphate: 2.6 *M*; 3.03 *M*; 3.25 *M*; 3.57 *M*; 3.68 *M*; 3.9 *M* and 4.1 *M*, by the addition of either saturated ammonium sulphate solution (containing 1 m*M* EDTA and adjusted to pH 7.0 with NH_4OH) (fractions P_1 to P_5) or solid ammonium sulphate (Fractions P_6 and P_7). The concentration of the saturated ammonium sulphate solution was taken as 3.9[9], since the fractionations (with the exception of the last one) were performed at 0–5 °C. The concentrations listed above correspond, therefore, to 66; 77.6; 83.3; 92; 94.3; 100% (at 0 °C) and 100% (at 25 °C) saturation respectively. In all cases the addition of the ammonium sulphate was made slowly and with efficient stirring. After an additional 10 min stirring period, the suspensions were centrifuged for 20 min at 37,000 × *g*. The bulky red precipitates obtained from the first and second fractions were washed with an $(NH_4)_2SO_4$-NaCl solution of the appropriate concentration, centrifuged again, and the washings pooled with the original supernatant fluids. All precipitates were redissolved in 1 ml of the *Tris*-HCl- 5 *M* NaCl- EDTA solution and used as such for the enzyme assays. In some of the experiments the saturated ammonium sulphate solution contained 0.3 m*M* NADH. For the experiments with solid ammonium sulphate, the cell-free extract was dialysed overnight against 100 volumes of 0.05 *M* *Tris*-HCl buffer (pH 7.6) containing 2 *M* $(NH_4)_2SO_4$ and 1 m*M* EDTA, and then the dialysed extract was brought to the same ammonium

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Fractionation of a cell-free extract from *H. cutirubrum* with saturated solution of ammonium sulphate

Fraction		Crude extract	P_1	P_2	P_3	P_4	P_5	P_6	P_7
Concentration of ammonium sulphate (*M*)		0	2.60	3.03	3.25	3.57	3.68	3.90	4.10
Volume (ml)		1.5	1.5	1.3	1.0	0.95	1.0	1.0	1.0
Protein (mg)		153	60	37	7.9	11.9	1.4	2.6	2.7
NADH oxidase	Total U	1.944	0.984	0.484	0.064	0.128	0	0	0
	Spec. act.	0.013	**0.016**	0.013	0.008	0.011	0	0	0
ME	Total U	1.254	0	0.806	0.621	0.320	0	0	0
	Spec. act.	0.008	0	0.022	**0.079**	0.028	0	0	0
GDH	Total U	12.480	0.960	2.640	6.240	1.337	0	0	0
	Spec. act.	0.082	0.016	0.070	**0.794**	0.113	0	0	0
GluDH	Total U	40.800	2.880	1.664	1.120	8.432	2.480	9.120	3.080
	Spec. act.	0.266	0.048	0.045	0.141	0.710	1.770	**3.493**	1.361
MDH	Total U	40.861	1.680	2.944	0.976	6.832	1.610	8.800	3.648
	Spec. act.	0.267	0.027	0.079	0.123	0.576	1.136	**3.343**	1.344
CS	Total U	7.720	0.662	0.904	0.008	1.882	0.294	1.801	0.544
	Spec. act.	0.050	0.011	0.025	0.001	0.158	0.210	**0.683**	0.198
GOT	Total U	1.704	0.021	0.064	0.064	0.152	0.128	0.256	0.410
	Spec. act.	0.011	0.001	0.002	0.008	0.013	0.091	0.097	**0.148**

The enzymes were assayed in a Beckman DB-G recording spectrophotometer at 30 °C, at 340 nm or 412 nm (for citrate synthase). The reactions were always started by addition of the enzyme solution. The reaction mixtures contained, in a final volume of 1 ml, the following amounts (in micromoles): NADH oxidase: *Tris*-HCl, 40; NADH, 0.15; KCl, 3,360; malic enzyme (ME): *Tris*-HCl, 40; $MnCl_2$, 1; NADP, 0.1; L-malate, 5; NH_4Cl, 1,000; glycerol dehydrogenase (GDH): *Tris*-HCl, 31; dihydroxyacetone, 2; NADH, 0.15; KCl, 3,300; glutamate dehydrogenase (GluDH): *Tris*-HCl, 11; α-ketoglutarate, 2; NADPH, 0.15; NH_4Cl, 80; NaCl, 1,000; malate dehydrogenase (MDH): *Tris*-HCl, 31; oxalacetate, 0.5; NADH, 0.15; KCl, 1,000; citrate synthase (CS): *Tris*-HCl, 40; oxalacetate, 0.5; acetyl-CoA, 0.15; 5.5′ dithiobis (2-nitrobenzoic acid) (DTNB), 0.1; KCl, 3,000; glutamate oxalacetate transaminase (GOT): *Tris*-HCl, 30; L-aspartate, 12.5; α-ketoglutarate, 5; NADH, 0.15; NH_4Cl, 1,000; pyridoxamine phosphate, 30 μg; pig heart malate dehydrogenase, 5 μg. The pH of the reaction mixture was 7.6 in all cases. Enzyme units (U) are expressed as μmoles/min; specific activities (spec. act.) are expressed as μmoles/min/mg of protein.

sulphate concentrations listed above, by the additions of solid salt in all cases. The fractionation of the cell-free extract in the *Tris*-HCl- 5*M* NaCl- EDTA solution by addition of solid ammonium sulphate was not possible.

The results of a typical fractionation are shown in the Table. NADH oxidase was assayed as a marker of the particulate material, and followed closely the contents of the red carotenoid pigment characteristic of the *Halobacteria*. 70% of the protein present in the crude extract, containing most of the NADH oxidase activity, was precipitated by ammonium sulphate concentrations up to 3.25*M* (P_1, P_2 and P_3). The latter 2 fractions also contained most of the ME and GDH activities. CS, MDH and GluDH, on the other hand, were recovered in the remaining fractions, which contained 12% of the protein present in the crude extract. Most of the GOT activity was recovered in P_6 and P_7. The order of precipitation by ammonium sulphate followed closely the order of elution from a Sephadex G-200 column (not shown), which is directly related to the molecular weights of the proteins, namely NADH oxidase, ME, GDH, GluDH, MDH, CS and GOT. In spite of the fact that no attempts were made to purify any of the enzyme in particular, good purifications and yields were obtained, namely 9.4-fold with 49% yield for EM (P_3); 9.6-fold with 50% yield for GDH (P_3); 13-fold with 22% yield for GluDH (P_6); 12.5-fold with 22% yield for MDH (P_6); 13.5-fold with 23% yield for CS (P_6) and 13-fold with 24% yield for GOT (P_7), calculated from the data in the Table. The reproducibility of the fractionation, when tested on different cell-free extracts, was good. When 0.3 m*M* NADH was added to the saturated ammonium sulphate solution (not shown in the Table) both GDH and MDH precipitated at a higher $(NH_4)_2SO_4$ concentration, with some improvement in the purification attained. The distribution pattern of the other enzymes and total protein was not affected by NADH.

When another aliquot of the cell-free extract used for the experiment shown in the Table was dialysed against a *Tris*-HCl- 2*M* $(NH_4)_2SO_4$ – EDTA solution, and then fractionated by addition of solid ammonium sulphate, the distribution pattern of the enzymes was different, resulting in slightly higher yields with lower purification values. This behaviour was probably due to the absence of NaCl during the latter fractionation. In addition, the changes in volume were much smaller when solid salt was used, resulting in different protein concentrations during the fractionation.

The ammonium sulphate fractionation performed by addition of saturated solution, as described in this communication, allows the simultaneous purification of several halophilic enzymes, with purifications and yields which are of the same order as those obtained in some cases by precipitation in the presence of protectors at low salt concentrations[4,5]; in the latter case, in general only one enzyme can be purified at a time, the others being denatured. When compared with the purification in the salt-free state, followed by renaturation of the enzyme by dialysis against 5*M* NaCl[3,4], the present method offers the advantage that the enzymes keep their native configurations throughout the purification procedure, resulting in higher yields. We have not been able to purify some of the enzymes studied here by any of the alternative methods mentioned. Thus ME was not recovered by dialysis of a salt-free preparation against 5*M* NaCl, nor was it protected by its substrates and cofactors (VIDAL and CAZZULO, unpublished observations). Similarly, we have been unable to recover GDH and GluDH from the salt-free state.

When compared with the fractionation with solid ammonium sulphate starting with a cell-free extract in 2*M* $(NH_4)SO_4$[6], the present procedure seems to allow a better purification, and is easier to perform and standard-

ize. The presence of NaCl during the fractionation does not interfere, since it is diluted by the addition of the saturated ammonium sulphate solution. The possibility of altering the fractionation pattern by the addition of a ligand specific for an enzyme or group of enzymes, as exemplified here with NADH for GDH and MDH, may be used to improve the purification attainable with ammonium sulphate alone.

We believe that the simple procedure outlined here can be useful as a general first step in the difficult task of the purification of halophilic enzymes[10].

Resúmen. Seis enzimas de la bacteria halófila extrema *Halobacterium cutirubrum* han sido parcialmente purificadas por fraccionamiento de los extractos crudos en ClNa 5 *M* con solución saturada de sulfato de amonio. Se discuten las ventajas del procedimiento, como un método general para enzimas halofílicas, comparado con otros métodos descriptos en la literatura.

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Determination of the Trapped Volume in a Pellet of Red Blood Cells

Separation of cells from a suspension by repeated centrifugation and resuspension, known as 'washing', is often inadequate. The volume trapped between sedimented cells after centrifugation is a measure of the degree of separation (of the cells) from the suspension medium. Knowledge of this value permits appropriate corrections and the relation between various measurable properties, and the cell volume, becomes possible. Trapped volume values reported in the literature, range from 0.5 to 10%, corresponding to various experimental systems, separation techniques and methods of measurement[1-5]. The rapid and rather accurate differential flotation method (DF)[1] is particularly useful when kinetics are studied with radioactive tracers[6].

In the present study, the volume trapped between red blood cells after DF separation was evaluated and compared with the volume trapped after ordinary centrifugation, using I^{131} serum albumin as tracer. The adsorption of I^{131}-labeled serum albumin, the transport of inorganic iodine across the cell membrane and hemolysis due to manipulation throughout the experiment, are accounted for.

Materials and methods. Blood was withdrawn from rabbits which received weekly injections of about 3 μCi of F_e^{59} per rabbit, over a period of 10 weeks. It was heparinized and diluted (1:1) with saline (0.16 *M* NaCl, 0.005 *M* KCl, 0.005 *M* phosphate buffer solution at pH 7.3).

Labeled human serum albumin (HSA) was added to that suspension to a final concentration of about 6 μCi/ml. The cells were then separated from the suspension medium and leucocytes by the DF method[1,7]. In this method cells are passed through phtalate ester solutions (Miles Yeda, Israel) of lower density than that of the cells. After centrifugation, a layer of water non-miscible phtalate solution separates the cells from the suspending medium. Hematocrites were obtained, and the relation between pellet volume and the radioactivity of F_e^{59} hemoglobin was established.

The DF separations were carried out in 0.4 ml polyethylene tubes. Each tube contained 0.05 ml of the separating fluid to which 0.1 ml of the suspension was added. Following centrifugation for 5 min at about 10,000 *g* the tubes were cut through the separating fluid, close above the pellet, with a razor blade. The radioactivity of the separated supernatant and pellet was then determined. An experiment in which erythrocytes were separated by ordinary centrifugation, was carried out for comparison purposes. A sample of blood diluted with saline was separated by the DF method, and another sample of the same blood was separated simultaneously, by ordinary centrifugation, in capillaries. After the separation, the capillaries were deep frozen for about 10 min and later cut with a glass knife through the pellet, so that contamination with the supernatant was avoided. Radioactivity of F_e^{59} hemoglobin served for evaluation of erythrocytes volume.

From radioactivity measurements of samples removed from the central portion of the separating fluid, it was found that the radioactivity transfer from the plasma-saline supernatant to the pellet, resulting from residual separating fluid, was less than 1/500% of the activity in the supernatant, and could be neglected.

Estimation of errors. Errors resulting from adsorbtion of I^{131}-HSA on the surface of the cells and possible transport of I^{131} through the cell membranes, were estimated as follows: Aliquots of packed cells, were added to (pipettes, previously wetted with saline were employed) plasmasaline solutions containing various concentrations of I^{131}-HSA: a) none, b) 0.04 μCi/ml, c) 0.15 μCi/ml. Samples of 0.1 ml were then separated by the DF method as above, the radioactivity of the pellets measured, and the changes (δ) in the I^{131}-HSA concentration of the plasma-saline solutions, following the removal of the cells, were determined.

From the dependence of (δ) on the original I^{131}-HSA concentration (C) of the plasma-saline solutions, (Figure 1A), that concentration for which $\delta \leq |\varepsilon|$ could be determined by interpolation. $|\varepsilon|$ is a number smaller than the value of the I^{131}-HSA concentration, expected from the dilution of the trapped volume by the plasma-saline solutions. When C is chosen so that $\delta \leq |\varepsilon|$, the I^{131} concentration of the pellet represents the contribution

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