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ADVANTAGES OF POTASSIUM BROMIDE GRADIENTS FOR ISOPYCNIC CENTRIFUGATION OF PROTEINS

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SUMMARY - The distribution of four enzymes(β-galactosidase, catalase,acid phosphatase and phenylalanine ammonia lyase) centrifuged to equilibrium is 40% narrower in potassium bromide gradients than in cesium chloride gradients.This is due to an increase of the apparent specific volume of proteins in potassium bromide.When a mixture of deuterium-labelled and unlabelled phenylalanine ammonia lyase is centrifuged in KBr gradients,the relative band widening is 5 to 6 fold larger than that obtainable in CsCl gradients,thus showing a considerably better resolution of the deuterated and normal proteins.The consequent advantages of KBr gradients for density labelling studies are discussed.

INTRODUCTION - Density labelling is one of the methods used to study the regulation of protein synthesis(1,2,3,4) and theoretical considerations of the labelling technique have been outlined(4,5).Two parameters are used to interpret the results of the density gradient profile of a labelled macromolecule:the shift in buoyant density and the bandwidth at half peak height(i.e;,the width of the profile at half maximum concentration distribution).Since in most cases it is not possible to separate completely native and labelled proteins,the knowledge of bandwidth is more useful than that of the buoyant density shift for bandwidth changes reflect the extent to which the molecular population has been labelled.However,CsCl gradients which have been used extensively,yield a poor resolution between"light" and

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heavy proteins.Surprinsigly,although it is known since 1961(6)that CsCl is not the more suitable salt for isopycnic centrifugation of proteins,few authors used other salt gradients(7,8).We report here a comparative study of isopycnic centrifugation of four enzymes in CsCl and KBr gradients.

MATERIALS AND METHODS

SOURCE OF ENZYMES-Seeds of radish(Raphanus Sativus, cultivar Longue Rave Saumonnée)were sown on moist filter paper and germinated at 25°C for 36h in the dark.They were then transfered to continuous standard far red light for 24h(9).For density labelling $[^2H_2]O$ (100%) was supplied during the light treatment .200 cotyledons were harvested and ground up in a mortar in 0.1M ammonium acetate buffer pH6.After centrifugation(20min,10000g) the crude extract was desalted through a Sephadex G50 column and partial purification was carried out on Sepharose 4B coupled to L-phenylalanine (10).Acid phosphatase and phenylalanine ammonia lyase were bound at pH6 and eluted at pH7.9 in 0.05M borate buffer.β-galactosidase(E.Coli) and catalase (bovine liver) were purchased from SIGMA.

ASSAY OF ENZYMES-Phenylalanine ammonia lyase (EC.4.3.1.5.) was assayed as follows:to each fraction from gradients,3ml of 0.05M borate buffer,pH8.8 containing L-phenylalanine(IOmM) were added.After incubation at 30°C for 24h the increase in absorbance at 290nm was measured.Acid phosphatase(EC.3.1.3.2.) activity was determined in 0.1M acetate buffer,pH5.2 using para nitropheyl phosphate as substrate(11).Catalase (EC.1.11.1.6) was assayed in 0.1M phosphate buffer,pH7(12);IOμl hydrogen peroxide were injected into the cuvette of the spectrophotometer and the decrease in absorbance at 240nm recorded for 30sec.β-galactosidase(EC 3.2.1.23)activity determination was carried out in 0.1M phosphate buffer,pH7 containing 0.05% o-nitrophenyl β-D galactopyranoside.After incubation(10 to 30min at 30°C)the reaction was stopped by addition of 5% sodium carbonate and the absorbance at 410 nm measured.

DENSITY GRADIENT PREPARATION-Cesium chloride gradients were prepared as follows:2.0g CsCl were dissolved in 2.0ml 0.05M borate buffer,pH8.8 for phenylalanine and acid phosphatase or in 2.0ml 0.1M phosphate buffer,ph7 for catalase and β-galactosidase.2.4ml of enzyme extract in the same buffers were then layered onto the CsCl solution.Two equivalent procedures were used for KBr gradients:either 1.5ml enzyme extract were layered on to 6ml of a saturated KBr solution or 3.5g KBr were dissolved in 7.5ml of extract(for dilute samples).Except for catalase,β-mercaptoehanol(10mM) was added in the centrifuge tubes which were then filled with liquid paraffin.

ISOPYCNIC CENTRIFUGATION -Isopycnic centrifugations were carried out in a BECKMAN L5 75 ultracentrifuge using a

type 75 Ti fixed angle rotor at 45,000rpm and 4°C for 40h (CsCl gradients) or at 52,000rpm and 6°C for 24h(KBr gradients) On completion of the run,two drop fractions were collected. Every tenth fraction was used to determine refractive index in an ABBE 60 refractometer and the values where then converted into density units(6,13).

RESULTS AND DISCUSSION - As shows figure 1,the distribution of the four enzymes was consistantly 36-40% narrower in KBr gradients than in CsCl gradients.The decrease in bandwidth was 36% for β-galactosidase,40% for catalase,37% for acid phosphatase and 38% for phenylalanine ammonia lyase.Buoyant densities were different from those obtained for CsCl gradients, which indicates that the interactions of proteins with KBr or CsCl are not the same.

According to Meselson et al(14) the standard deviation of the gaussian concentration distribution σ of a macromolecule in a salt gradient can be written: $\sigma^2 = \frac{RT}{M\bar{\nu}(d\rho/dr)_r \omega^2 r}$ |1|

where R is the gas constant,M is the molecular weight of the macromolecule,$\bar{\nu}$ is the apparent specific volume of the macromolecule,$(d\rho/dr)_r$ is the slope of the gradient at the distance r(radial distance of the centre of the distribution) and ω is the angular velocity.Considering this equation,our results can be explained by differences of the apparent specific volume of proteins in KBr and CsCl gradients. Indeed,if it was not so,the bandwidth should be 1.3 fold larger in KBr than in CsCl.This can be calculated from |1|, the slope of the gradient being $0.0061 g.ml^{-1}$ for KBr and $0.0135 g.ml^{-1}$ for CsCl.Therefore we can assume that the narrower distribution of proteins in KBr gradients is due to a higher apparent specific volume of the macromolecules in this salt.

Since for a given salt gradient,the resolution is independent of angular velocity(6),an improvement in resolution between

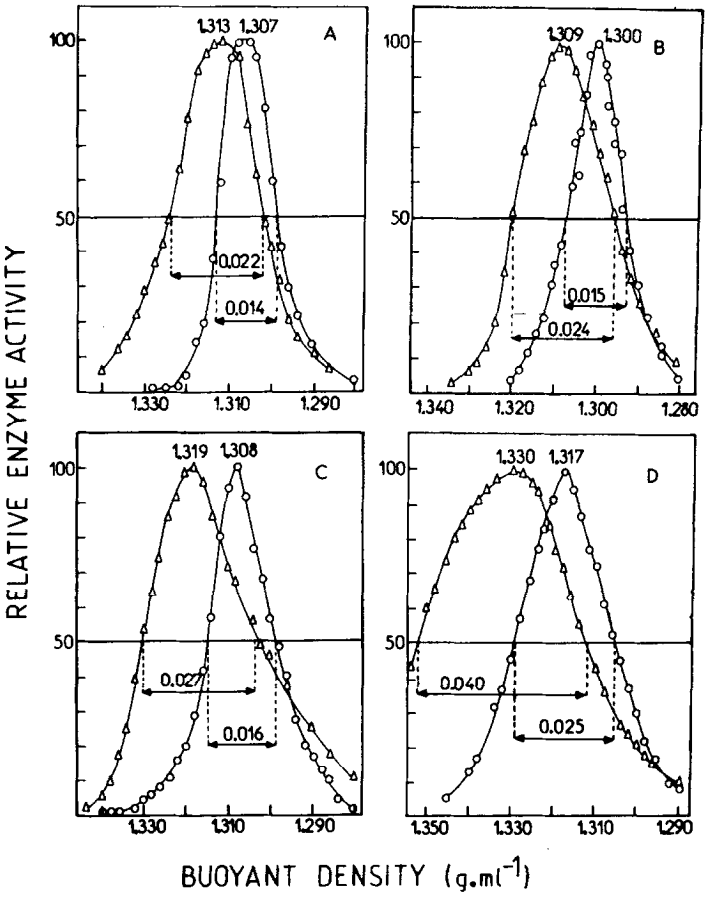

Figure 1.Comparative distribution of some native proteins in CsCl and KBr gradients.Isopycnic centrifugation of (A) β-galactosidase;(B) phenylalanine ammonia lyase;(C)catalase and(D.)acid phosphatase in CsCl(Δ)orKBr gradients(○). Enzyme activities were first plotted versus fraction number and then versus density values.Horizontal bars represent the bandwidth measured at half peak height and expressed in density units(g.ml^{-1}).Profiles are the mean of two separate experiments.

two macromolecules will be obtained by using the more suitable salt.The solute to be used should satisfy the following conditions:a)the macromolecules under investigation must band in the linear zone of the gradient and b)in case of enzyme study ,the enzyme must be stable in the salt used.Concerning the latter condition,Hu et al(7) reported that β-galactosidase was unstable in KBr.We examinated the stability of the four

Table 1.Stability of some enzymes in KBr and CsCl.Enzymes were incubated with KBr or CsCl at the same concentration that the one used for isopycnic centrifugations.Concentration of β-mercaptoethanol was 10 mM.Activity was measured after 6 and 24 h of incubation at 5°C and results expressed as percentage of initial activity.

	incubation time (h)	CsCl	CsCl + β-mercaptoethanol	KBr	KBr + β-mercaptoethanol
catalase	6	52	6	47	20
	24	50	0	40	3
acid phosphatase	6	85	87	87	88
	24	72	77	75	80
phenylalanine ammonia lyase	6	62	88	51	80
	24	50	62	44	66
β-galactosidase	6	80	90	30	54
	24	56	70	10	45

enzymes in KBr and CsCl(table 1).Only β-galactosidase was significantly more labile in KBr.However it was possible to prevent the enzyme from a drastic loss of activity by means of β-mercaptoethanol.Furthermore since centrifugation time was shorter for KBr gradients (because of the higher speed) and that enzyme denaturation is a function of time,the final activity was similar for both gradients.

The resolution obtainable between two macromolecules in a density gradient has been defined by the ratio $\Delta r/(\sigma_1+\sigma_2)$ (6) where Δr is the distance between the two centres of the gaussian distributions and σ_1,σ_2 the standard deviation of the distribution of the two species.Use of KBr instead of CsCl lead to an increase of the upper term of the ratio (Δr)since the slope of KBr gradients is shallower,whereas($\sigma_1+\sigma_2$) decreases and,hence, the resolution is much better with KBr than with CsCl.This is illustrated by the following experiment: phenyalanine ammonia lyase was extracted from cotyledons of radishes grown either on H_2O or $[^2H_2]O$ and the same amount (in terms of activity)of"light" and "heavy" enzymes were centrifuged together.Figure 2 shows that the percentage increase in bandwidth of the mixture compared with that of the native protein was much greater with KBr gradients.This increase was 16% for CsCl gradients and 90% for KBr gradients.In density labelling experiments,an increase in bandwidth for a given protein indicates that the molecular population is heterogeneous(i.e.,a mixture of labelled and native protein). Then it is possible to estimate protein turnover from data of a time course labelling experiment.However the increase in bandwidth being usually small(10% or less) with CsCl gradients (1,3,4,11,15,16) it is somehow difficult to interpret unambiguously the results.

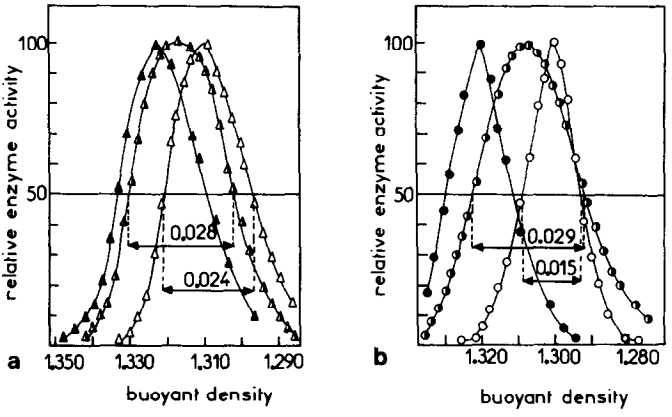

Figure 2.Comparative distribution of mixed native and $|^2H_2|$ labelled phenylalanine ammonia lyase in CsCl and KBr gradients. Isopycnic centrifugation of a mixture (1/1) of native and deuterated phenylalanine ammonia lyase in CsCl(triangles) or Kbr(circles);solid symbols:labelled enzyme;open symbols:native enzyme;half solid symbols:mixture.Horizontal bars represent bandwidth values.Profiles are the mean of two separate experiments.

Provided that the required conditions for KBr use are satisfied(range of buoyant density and stability of the enzyme)this technique can be applied profitably to protein turnover determinations,making them more reliable.Furthermore since a higher speed can be used for KBr gradients,the time required to reach equilibrium is reduced and therefore the cost of each experiment is reduced;besides more runs can be carried out within a given time.

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REFERENCES

1. Filner,P and Varner,J.E.(1967)Proc.Nat.Acad.Sci.USA. 58,1520-1526.
2. Longo,C.P.(1968)Plant Physiol.43,660-664.
3. Williams,L.S. and Neidhart,F.C.(1969)J.Mol.Biol.43,529-550.
4. Acton,G.J.,Drumm,H.and Mohr,H.(1974)Planta 121,39-50.
5. Lamb,C.J. and Rubery,P.H.(1976)Biochim.Biophys.Acta. 421,308-318.
6. Ifft,J.B.,Voet,D.H. and Vinograd,J.(1961)J.Phys.Chem. 65,1138-1145.

7. Hu,A.S.L.,Bock,R.M. and Halvorson,H.O.(1962)Analyt.Biochem. 4,489-504.
8. Boudet,A.,Humphrey,T.J. and Davies,D.D.(1975)Biochem.J. 152,409-416.
9. Huault,C.(1974) Plant Sci.Lett.3,149-155.
10. Blondel,J.D.,Huault,C.,Faye,L.,Rollin,P. and Cohen,P.(1973) FEBS Lett.36,239-244.
11. Johnson,C.B.,Attridge,T.H. and Smith,H.(1975)Biochim.Biophys. Acta,317,11-19.
12. Chance,B.and Herbert,D.(1950)Biochem.J.46,402-410.
13. Vinograd,J. and Hearst,J.E.(1962)Fortschr Chem.Org. Naturstoffe 20,372-378.
14. Meselson,M.,Stahl,W. and Vinograd,J.(1960)Proc.Nat.Acad.Sci. USA.43,581-588.
15. Attridge,T.H.,Johnson,C.B. and Smith,H.(1974)Biochim. Biophys.Acta 343,440-451.
16. Wellmann,E. and Schopfer,P.(1975)Plant Physiol.55,822-827.