# THE DEUTERATION OF THE HISTONES OF PHYSARUM POLYCEPHALUM AS A STEP TOWARDS THE PREPARATION OF SELECTIVELY DEUTERATED NUCLEOSOME CORE PARTICLES

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ABSTRACT

Physarum polycephalum can be grown in shake flask culture where all of the peptone component of the feed stock has been replaced by a deuterated algal hydrolysate. These conditions give approximately 35% overall deuterium incorporation into the basic nuclear proteins. This level of incorporation, together with the maintenance of a reasonable maximum growth, show P. polycephalum to be a convenient system for the preparation of nucleosome core particles with selectively deuterated histones. Selectively deuterated core particles offer considerable advantages over uniformly protonated ones when structural studies using neutron scattering are carried out.

A combination of proton and deuterium magnetic resonance spectroscopy shows that under the conditions so far studied the deuterium incorporation is not uniformly distributed throughout the amino acid residues of the proteins. A 60% deuteration of the terminal apolar methyl groups is accompanied by only 28% deuteration of the hydrogen atoms bound to the α-carbon.

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Our observation that α-carbon hydrogens are exchanged in vitro reactions with $^{2}H_3O^+$, albeit at elevated temperatures, could well indicate these hydrogens are exchanged in vivo with the solvent water of the growth medium. Suggestions are made as to other possible variations in feed stock components which could lead to a higher overall level of deuterium incorporation.

Deuteration - Physarum polycephalum - neutron scattering - histones - nucleosome core particles.

## INTRODUCTION

A steady increase in the use of neutron scattering as a technique for investigating structural problems involving biological macromolecules has been observed over recent years. Further, the improvements in instrumentation (1) which have recently come into operation, together with the possibility of a pulsed neutron source becoming operational in the United Kingdom, should see even more interest being generated.

The strength of the neutron scattering technique lies in the fact that the scattering length densities of all biological macromolecules lie within those values for $^{1}H_2O$ ($-0.56 \times 10^{10} cm^{-2}$) and $^{2}H_2O$ ($+6.34 \times 10^{10} cm^{-2}$). This means that by choosing the correct ratio of $^{1}H_2O$ : $^{2}H_2O$ as solvent, the scatter from a particular biological macromolecule can be contrast matched to that solvent (2). It also permits, in solvents with a high percentage of $^{2}H_2O$, for the neutron scatter to be studied in reverse contrast. Thus, in biological complexes, where the individual components have different scattering length densities, it is possible to examine the scatter from certain components in the sample complex while contrast matching the scatter from others.

However, if the multicomponent system contains molecules of similar composition the scattering length densities will also be very similar and contrast matching of just one specific species by the solvent will not be possible. This problem, in contrast matching, can be overcome if a selected molecule or molecules in the complex are present in a deuterated form (3).

The replacement of hydrogen by deuterium will increase the positive scattering length density of the macromolecule. Consequently, if the extent

of deuteration is sufficient, when the non-deuterated macromolecules are contrast matched to the solvent, the observed neutron scatter will be derived predominantly from the deuterated components. Similarly, if the solvent contrast matches the deuterated component the scatter will originate predominantly from the protonated entities. (See Fig 1).

The structure of the nucleosome core particle, a fundamental sub-unit of all eukaryotic chromosomes continues to create considerable interest as it is obviously implicated in the processes of replication and transcription. The present accepted low resolution structure is of 145 bp of DNA wrapped around two molecules each of the histones H2A, H2B, H3 and H4 and having the overall dimension of a cylinder, height 5.5 nm and diameter 11 nm (4,5,6). However, to date there is no precise information as to the relative positions of the histone fractions within the nucleosome, but we believe that if specific histone fractions could be selectively deuterated and the complex studied by neutron scattering (2,7), this information would become available. To this end we have investigated the slime mould Physarum polycephalum as a system for producing deuterated histone proteins. The choice of P. polycephalum for our objective was influenced by the published reports (8,9) for the isolation of core particles from the nuclei of this slime mould, and being grown in shaken liquid culture, it readily allows for the introduction of labelled compounds into the growth medium.

## EXPERIMENTAL

### Hydrolysis of deuterated algae

Dry deuterated algae were obtained from Spectrometrie Spin et Techniques, Paris, France and hydrolysed according to Putter et al (10). This technique involved the removal of pigment by washing in ethanol and diethyl ether, hydrolysis with 6M HCl at $110^{o}C$ and ion exchange chromatography to separate the amino acids from other cell debris.

## Growth Media

Physarum strain $M_3C$, was grown at $26^oC$ in shake flask culture containing 100 $cm^3$ of semi-defined medium per flask. (Semi-defined medium per 100 $cm^3$ of liquid : peptone 400 mg, glucose 1000 mg, yeast extract 150 mg, $KH_2PO_4$ 200 mg, $ZnSO_4 . 7H_2O$, 3.4 mg, $FeCl_2 . 4H_2O$, 6 mg, citric acid 354 mg, EDTA, disodium salt 22.4 mg, $CaCl_2 . 2H_2O$, 90 mg, $MgSO_4 . 7H_2O$, 60 mg. In various experiments the 400 mg of peptone was replaced by (i) 100 mg of peptone, (ii) 400 mg of hydrolysed algae, (iii) 350 mg of hydrolysed algae plus 50 mg of peptone.

## Estimation of the Growth of Physarum Microplasmodia

1 $cm^3$ aliquots of microplasmodia were withdrawn at various time intervals from the shaken culture flasks using a wide mouth pipette. To ensure the 1 $cm^3$ sample was as representative as possible, flasks were vigorously agitated immediately prior to withdrawal. The microplasmodia were pelleted using an Eppendorf centrifuge, 5 min, top speed, and washed twice with 1 $cm^3$ aliquots of water using the same centrifugation conditions to produce the pellet in each case. Disruption of the pellet was carried out by thoroughly mixing with 1 $cm^3$ of saturated urea solution containing 2M NaCl and the mixture left for two hours with periodic vigorous agitation. Cell debris were then pelleted by centrifugation and 0.5 $cm^3$ of the supernatant treated with 2.5 $cm^3$ of 2M NaCl in saturated urea solution. The absorption at 260 nm was measured using a 1 cm path length cell with 2M NaCl saturated urea solution as reference. This measurement should be proportional to total nucleic acid.

All operations were carried out at $4^oC$ except for the sampling procedure and the spectroscopic measurement.

## Histone Extraction and Electrophoresis

Histones were extracted from Physarum nuclei with 1M $CaCl_2$ at $80^oC$ using essentially the method described by Mohberg and Rusch (11). Electrophoresis was carried out using 12% polyacrylamide gels containing 0.1% sodium dodecyl sulphate.

## Deuteration of Individual Amino Acids

5-6 mg of the amino acids L-leucine, L-lysine, L-alanine were dissolved in 2.5 $cm^3$ of 20% w/v $^2HCl$ (in $^2H_2O$) and heated in a glass tube under partial vacuum. The heating conditions and level of deuteration of the α carbon hydrogen atoms achieved are given in Table 1.

Table 1

Deuteration of the α C-H bonds in individual amino acids by heating with 20% w/v $^2HCl$ in $^2H_2O$.

| Amino Acid | Temp | Time | Result |
|---|---|---|---|
| L-lysine | 110°C | 16 hr | No effect |
| " | 145°C | 63 hr | 50% deuteration |
| L-alanine | 110°C | 16 hr | No effect |
| " | 145°C | 63 hr | 75% deuteration |
| " | 145°C | 127 hr | >95% deuteration |
| L-leucine | 110°C | 16 hr | No effect |
| " | 145°C | 63 hr | 60% deuteration |

## Determination of Deuteration Levels

### (i) Individual Amino Acids

The extent of deuteration of the individual amino acids L-leucine, L-lysine, L-alanine were measured by the relative losses in area of the proton megnetic resonance spectra. Spectra of the fully protonated individual amino acids, at a known concentration in $^2H_2O$ were used as standards.

### (ii) The Basic Proteins

The above method of estimating the extent of deuteration for the individual amino acids cannot be used directly for histone mixtures. The loss in area of proton resonance as an estimation of percentage deuteration requires a standard proton spectrum to which the partial deuterated product can be compared. However, extraction of histones from Physarum is not always achieved with a constant efficiency and the relative amounts of the individual histone

fractions can vary from preparation to preparation. The different histone fractions have a different amino acid composition and hence a standard protonated Physarum histone extract cannot be produced directly. Further, spectrophotometric determination of histone concentration is very susceptible to impurity errors.

These problems of composition and concentration were overcome by direct comparison of the peak areas of proton and deuterium magnetic resonance spectra of hydrolysed (6M HCl, 18 hr at 110$^{o}$C) protein samples. The spectra for both nuclei were recorded under conditions that could be directly compared. The same concentration for both spectra was achieved by removing the solvent $^{1}H_2O$ under reduced pressure from the magnetic resonance sample tube after recording the deuterium spectrum, and then adding an equal volume of $^{2}H_2O$ before recording the proton magnetic resonance spectrum. A mixture of protonated and deuterated methylene chloride in carbon tetrachloride was used as an external standard in order to match instrument settings for the production of spectra.

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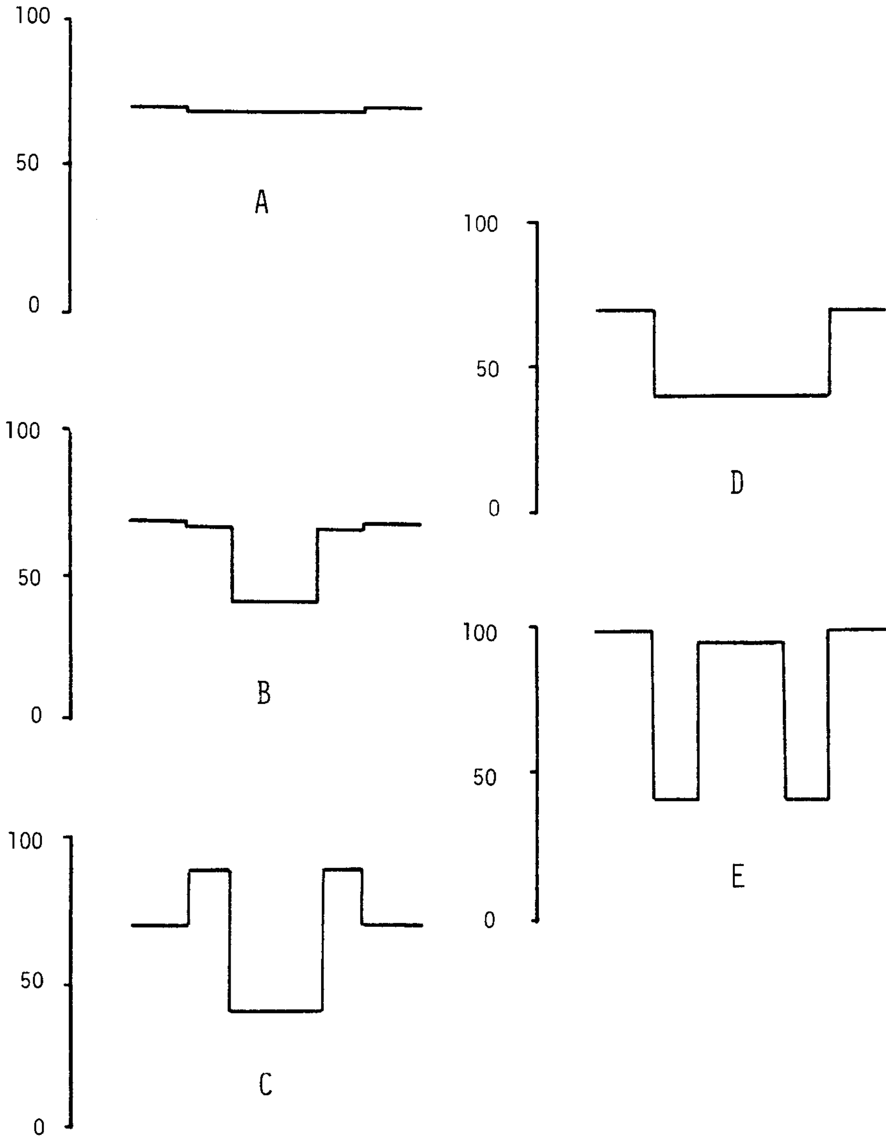
Figure I

Schematic representation of the contrast variation obtained for core particles with various deuterated components :

(A) $^{1}$H DNA + $^{2}$H (~40%) Histones H2A H2B H3 and H4.
(B) $^{1}$H DNA + $^{2}$H (~40%) Histones H2A+H2B $^{1}$H Histones H3+H4.
(C) $^{1}$H DNA + $^{2}$H (~80%) Histones H2A+H2B $^{1}$H Histones H3+H4.
(D) $^{1}$H DNA + $^{1}$H Histones H2A H2B H3 and H4.
(E) $^{2}$H (~95%) DNA + $^{1}$H Histone H2A+H2B $^{2}$H (~95%) Histone H3+H4.

The ordinate represents the equivalent solvent composition (as percentage $^{2}H_2O$ in the $^{2}H_2O/^{1}H_2O$ mixture) which contrast matches to the various core particle components. The core particle is represented as a symmetrical entity with the DNA on the outside, the histones H3 and H4 at the centre and the histones H2A and H2B in the intermediate positions.

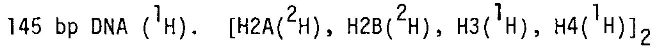
Figure 1



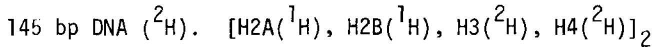
As $^{1}H$ and $^{2}H$ nuclear magnetic resonance spectra have similar chemical shifts, specific functional groups in the hydrolysed protein are easily recognised, and deuteration levels pertaining to these groups can be immediately calculated.

## DISCUSSION

To prepare nucleosome core particles with selectively deuterated histone pairs it is necessary to initially isolate core particles from both protonated and deuterated sources. Both types of core particle can then be selectively depleted of the histones H2A and H2B and reconstituted, but with the initially removed histones interchanged so that the final products should be :



145 bp DNA ($^{1}H$). $[H2A(^{2}H), H2B(^{2}H), H3(^{1}H), H4(^{1}H)]_2$



145 bp DNA ($^{2}H$). $[H2A(^{1}H), H2B(^{1}H), H3(^{2}H), H4(^{2}H)]_2$

A method for the complete and selective removal of histones from core particles and their subsequent reconstitution has recently been elucidated (12) using calf thymus and mastocytoma and there is every indication that the method can be extended directly to P. polycephalum.

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Figure 2

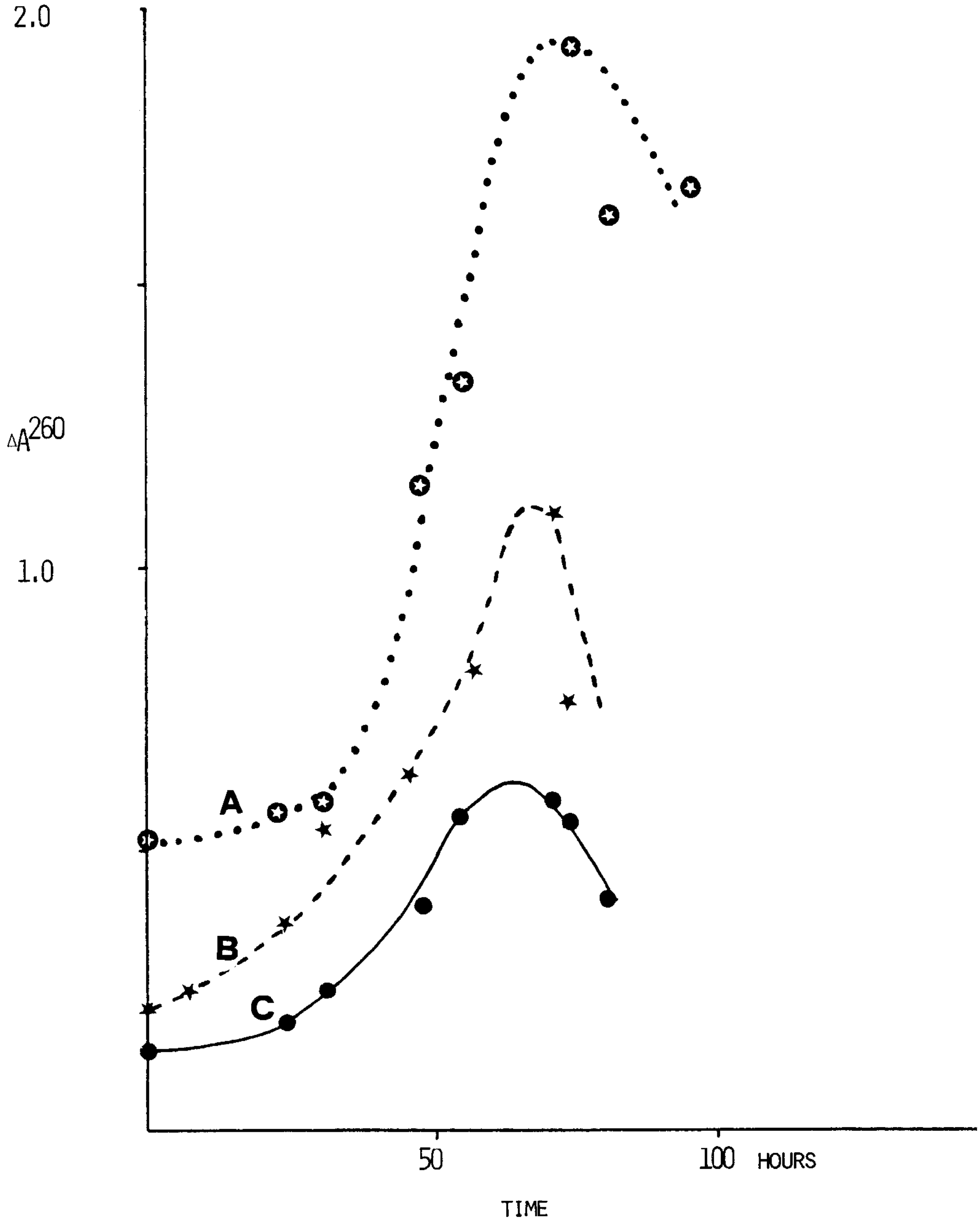
Growth curves of Physarum polycephalum.

(A) Standard growth, with 400 mg of peptone per 100 $cm^3$ of medium.

(B) The total peptone in the medium (100 $cm^3$) replaced by 400 mg of deuterated hydrolysed algae.

(C) Total peptone reduced to 100 mg per 100 $cm^3$ medium.

Figure 2



However,if the two histone fractions H2A and H2B were approximately 40% deuterated and the histones H3 and H4 protonated, then scatter in a 65% $^2H_2O$ solvent would come predominantly from the H3 and H4 histones. Experiments using core particles of this composition although being able to give useful information concerning the distribution of H3 and H4, require neutron scattering measurements of long duration in order to eliminate background scatter. A more desirable situation would be to obtain higher deuteration levels of the selected histone pairs H2A and H2B (Fig 1C) and to obtain the difference function between these partially deuterated core particles and the fully protonated ones (Fig 1C - Fig 1D). The solvent in all these experiments could remain at a composition which contrast matches to the DNA.

The situation where the DNA and histones H3 and H4 are highly deuterated and histones H2A and H2B are protonated is represented in Figure 1E. The scatter from this complex in both approximately 100% $^2H_2O$ and the difference function, Fig 1E - Fig 1D in approximately 40% $^2H_2O$ would also give valuable information.

The incorporation of deuterium into specific enzymes of P. polycephalum has been reported previously by both Mitchelson (15) and Huttermann (16,17,18). The work described by Mitchelson resulted in the deuteration of growth associated histone kinase in synchronously dividing single plasmodia. Such a system was not considered suitable for our purpose due to the very low yield of histone (approx $3x10^{-3}$g) per culture plate. This paper further reported that microplasmodia would not grow continuously in shake flask culture. However, Huttermann who has been concerned with studies involving the deuteration of glutamate dehydrogenase and glucose-6-phosphate dehydrogenase obtained deuterium incorporation in both continuously growing shake flask culture and during the induction of spherulation. Further, and importantly, the incorporation of deuterated amino acids did not appear to influence the activities of the derived enzymes, but was reported to interfere with general protein metabolism and synthesis (18).

Our experiments, as the initial step towards obtaining specifically deuterated core particles, were concerned with both the levels of histone deuteration that could be achieved and the total yield when protonated growth medium components were replaced by deuterated analogues. The obvious replacement of the biological peptone in the semi-defined medium with $^2$H-amino acids was tempered by cost and a deuterated algal hydrolysate was used instead. The results, using the total nucleic acid content as an estimation of growth, show little change in the growth characteristics with respect to rate and yield when up to 87% of the protonated peptone is replaced by the deuterated algal hydrolysate. As an indication, prior to deuterium analysis, that the Physarum was metabolising the deuterated hydrolysate we grew one culture with just 100 mg of peptone in the feed stock instead of the standard 400 mg. Only 1/3 of the expected total growth was obtained. However, increasing the deuterium content of the growth medium further by replacing all of the peptone with 400 mg of algal hydrolysate had a detrimental effect on the total growth; reducing it by about 30% (Fig 2).

The basic proteins, whether fully protonated or partially deuterated, extracted from the Physarum microplasmodia had similar (Fig 3) electrophoretic migration patterns,and the yield from a fully grown culture using 100 $cm^3$ of medium was approximately 1 mg. Ten such cultures should yield 3-4 mg of the histone pair H2A+H2B, a quantity sufficient for core particle reconstitution and neutron scattering experiments.

Our method for estimating deuteration levels involved a comparison of proton and deuterium magnetic resonance spectra of the extracted histone after the latter had been hydrolysed. This technique affords a direct comparison of the deuteration levels of specific groups of the constituent amino acids and as indicated in the experimental section of this paper, is not dependent on concentration measurements or the composition of the sample.

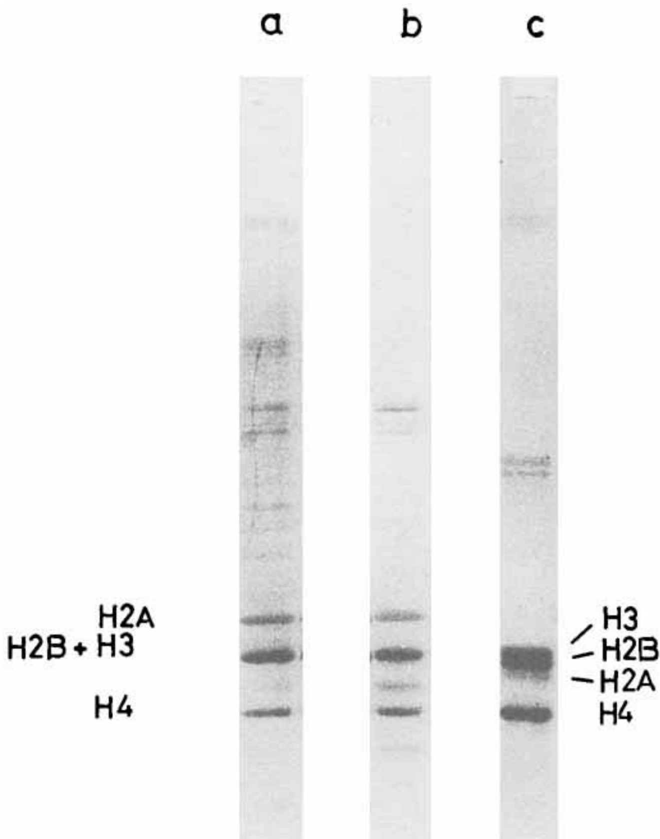



Figure 3

Polyacrylamide Gel Electrophoresis in 0.1% sodium dodecyl sulphate of (a) deuterated *Physarum* histones, (b) protonated *Physarum* histones, (c) calf thymus histones. The bands corresponding to the core particle histones are indicated in the figure, those assigned to the *Physarum* histones are according to Chahal (19).

The maximum deuteration we observed was in the histone extracted from the pooled microplasmodia of three shake flasks grown on media containing 400 mg of deuterated algal hydrolysate per 100 $cm^3$. Comparison of the proton and deuterium nmr spectra showed that 60% of the terminal methyl groups, $\delta$ ($^1$H) = 1.0-0.8, of the apolar amino acids were deuterated. If the determination was extended to include certain methylene groups of the apolar amino acids as well as similar groups of the basic amino acids lysine and arginine then the deuteration level was estimated at 43%. Although this high level of deuteration was found amongst the apolar amino acid residues, deuteration of the protons, recognised by their chemical shifts as being attached to the $\alpha$-carbon atoms, was much lower (28%). This obviously indicates that the metabolism in Physarum allows for a more ready exchange of the $\alpha$-carbon hydrogen while the hydrogen atoms of the apolar groups are much more strongly bound.

The difference in exchangability of the hydrogen atoms attached to the $\alpha$-carbon as compared to the other carbon bonded hydrogens, in amino acids has been observed by us (Fig 4) in vitro. Treatment of the amino acids L-leucine, L-lysine and L-alanine with 20% $^2$HCl in $^2H_2O$ at 145$^oC$ has in all cases shown considerable exchange of the $\alpha$-carbon linked hydrogen while the other carbon linked hydrogens are unaffected. However when the experiment was carried out at 110$^oC$ there was no perceptible change in the peak areas in the nmr spectra of the different chemically shifted hydrogens.

This latter observation is important in order to exclude that low levels of $\alpha$ C-H deuteration were not the result of deuterium $\rightarrow$ proton exchange during hydrolysis of either the deuterated algea feed stock or the extracted histones prior to analysis. Both these latter operations were carried out at 110$^oC$ with 6M HCl.

## CONCLUSION

These preliminary studies show that microplasmodia from Physarum polycephalum can be made to grow in shake flask culture where all of the biological peptone component of the medium is replaced by a deuterated algal hydrolysate. Although under these conditions the maximum microplasmodium growth expected is reduced by 1/3 it does allow an incorporation of approximately 60% deuterium into the apolar residues of the basic Physarum proteins. These results are very encouraging for the deuteration already achieved should be sufficient to carry out preliminary contrast matching neutron scattering studies when the appropriate core particles have been prepared.

To obtain a higher overall level, other components in the feed stock could well have to be present in a partially deuterated form or the culture medium formulation modified in some way. The citric acid, a component in the standard protonated medium used in this study, at a concentration of 0.35 g per 100 $cm^3$, is converted in the citric acid cycle to α-ketoglutarate and oxaloacetate. The α-ketoglutarate is a recognised precursor of the amino acids glutamate, proline and arginine, while the oxaloacetate is a metabolite in the formation of aspartate and thence methionine, lysine, threonine and isoleucine.
Protonated glucose present in 2½ times the concentration in the growth medium as the biological peptone (or deuterated algal hydrolysate), is also a possible factor which could lead to the lowering of deuteration levels. In addition it must be noted that two other amino acid precursors, 3-phosphoglycerate (serine, cysteine and glycine) and pyruvate (alanine, valine and leucine) are immediately derived from glucose.

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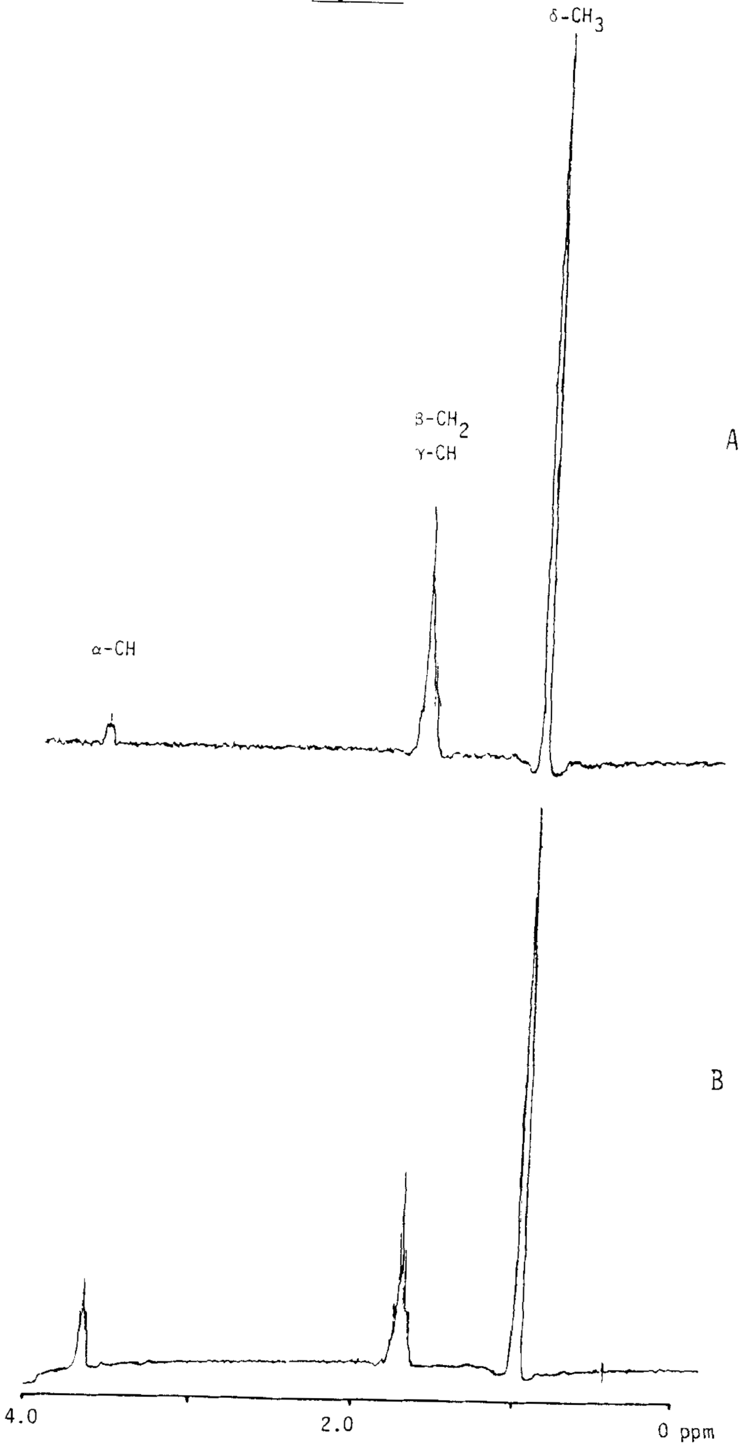
Figure 4

Proton magnetic resonance spectra in $^{2}H_3O^+$ showing (a) partial deuteration (approx 60%) of the αC-H of L-leucine, (b) fully protonated L-leucine.

The peak area ratios were estimated for (A) 0.4 : 3 : 6, (B) 1 : 3 : 6.

Figure 4



Most of the amino acids mentioned above are non-essential for the growth of Physarum microplasmodia. Hence amino acids metabolised from the protonated precursors glucose and citrate could well compete in the metabolic pool with the deuterated amino acids, supplied directly from the algal hydrolysate, for the incorporation into proteins.

An alternative to deuterated glucose in the growth medium may be provided by the use of protonated glycerol. A private communication (20) has indicated that complete substitution of glucose by glycerol in the culture medium has little effect on the growth rate of the microplasmodia. However, as glycerol can be phosphorylated and reduced to glyceraldehyde 3-phosphate,a metabolite in the glycolytic pathway, protonated amino acids may also result if this change is instigated.

If the solvent for the growth medium is changed from pure water to a $^{1}H_2O$-$^{2}H_2O$ mixture then partial deuterium incorporation would be an alternative to hydrogen in biosynthetic processes requiring molecular water as a metabolate. Experiments in this laboratory have shown that in 40% $^{2}H_2O$ the microplasmodia can be maintained in culture without inducing spherulation. Thus experiments could commence using this percentage $^{2}H_2O$ but the Physarum may well be adapted through a number of generations to grow in a higher $^{2}H_2O$ ratio.

The possibility of the yeast extract, analysed as containing 4% amino nitrogen, being a source of protonated amino acids should be greatly reduced if it is replaced by an equivalent mixture of biotin + thiamine (21).

As demonstrated earlier, the coupled technique of proton and deuterium magnetic resonance spectroscopy allows a ready and accurate determination of deuteration levels. Thus any increase or otherwise in the deuteration of specific amino acid residues, brought about by the changes in growth medium composition indicated above, will be immediately monitored and the growth conditions changed accordingly.

Such studies to obtain higher deuteration levels are now in progress. For neutron scattering studies the most advantageous situation would be to achieve complete deuteration of the core particle with respect to both histone and DNA such that they contrast match at a level equivalent to approximately 100% $^2H_2O$. Selective depletion of deuterated H2A and H2B histone followed by reconstitution using their 'normal' protonated analogues would then allow the latter to be studied by neutrons in maximum contrast.

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