The Choice of the Suspension Medium for Rat-Liver-Cell Nuclei

By J. ST L. PHILPOT AND JEAN E. STANIER

Medical Research Council Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell, Didcot, Berks

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The isolation and study of cell particles such as nuclei, mitochondria, etc., require a suspension medium which should be chosen so as to preserve intact the whole structure and function if possible, or at least the particular features being investigated. The natural medium, cell sap, is difficult to obtain free from extracellular fluid, and its composition is therefore largely unknown. Many artificial media have been advocated for nuclei (cf. reviews by Allfrey, Mirsky & Stern, 1955, and Dounce, 1954, 1955), and a surprising variety of abnormal appearances can be produced by conditions which are becoming increasingly well defined and which follow different rules for each abnormality. The present work is an attempt to define these conditions still better, and to design an aqueous artificial medium that satisfies several visual criteria of normality. These simple criteria alone suffice, without the aid of enzymic criteria, to exclude many of the media previously advocated, if complete normality is the aim.

The criteria of normality used in the present work are all based on observation with the phase-contrast microscope, and are as follows:

(1) The nuclei should be non-granular, i.e. their optical density should be uniform except for the nucleoli. This requires that the ionic strength of the medium should be below a critical value depending on the pH, the concentration of bivalent cations (e.g. Ca^{2+} and Mg^{2+}), the dielectric constant of the solvent, etc. (see Expt. 1). Moderate granularity seems to be reversible, and is therefore probably harmless. It may even be related to the condensation of chromosomes at mitosis. Nevertheless, for simplicity it is better avoided in experiments on resting nuclei.

(2) The nucleoli should be clearly visible and the nucleus should not be swollen (see Expt. 2). This sets a lower limit to the ionic strength, which is fortunately below the upper limit defined by criterion (1).

(3) The nuclei should not be clumped, even after being centrifuged and resuspended. The best way of satisfying this criterion seems to be by lowering the dielectric constant of the solvent and by keeping the ionic strength of the medium or the concentration of bivalent ions as high as is compatible with criteria (4) and (1) (see Expt. 3).

(4) The mitochondria should be sufficiently unclumped to be separable from nuclei by centrifuging. Mitochondrial, unlike nuclear, clumping increases with ionic strength and with concentration of bivalent ions, and there seems to be no known aqueous medium in which both kinds of clumping are completely prevented. The choice of ionic strength during the centrifugal separation is therefore determined by a careful balance between criteria (3) and (4).

(5) The nuclear membrane should be free from 'blebs': i.e. clear round bubbles attached locally to the membrane which apparently contain the medium in which they have formed, and which behave osmotically when transferred to another medium. Anderson (1953*a*), who first described blebs, found that they could be prevented by sucrose. We have found that glycerophosphate will also prevent them (see Expt. 4).

(6) When the nuclei are transferred to molar sodium chloride the deoxyribonucleic acid (DNA) should swell through the nuclear membrane, forming a halo, and thereby demonstrate that it is still present in soluble viscous form (see Expt. 5). The haloes are most easily seen in the presence of mitochondria or other small particles.

(7) The nuclear contents as judged by optical density should not diminish on standing. This can be prevented by sucrose, or better by glycerophosphate (see Expt. 6).

METHODS

Perfusion of liver. The livers of Wistar-strain rats anaesthetized with ether were perfused either via the portal vein through a glass cannula or via the left ventricle with a 30 ml. syringe, first with ice-cold 0.15M-NaCl until all the blood was washed out, and then with ice-cold 0.29M sucrose. 0.15M-NaCl was found to wash out blood more completely than sucrose (cf. Dounce, 1955), possibly by keeping plasma globulins in solution. It was subsequently washed out with sucrose so that it did not contaminate the homogenizing fluid. The whole operation took about 10 min. Perfusion through the left ventricle was quicker but somewhat less effective than perfusion through the portal vein. The liver was removed and kept on ice in 0.29M sucrose until it was needed. Storage in this way for 2 days did not alter perceptibly the appearance of homogenates, but except for preliminary experiments the perfused liver was always used within 4 hr.

Homogenization of liver. The liver was cut up with scissors and homogenized in a $\frac{1}{8}$ in. precision-bore glass tube closed by a rubber bung at the bottom, with a non-rotating stainless-steel conical plunger with a half-angle of 6° , widest at the bottom, where the diameter was 0.004 in. less than the bore of the tube (i.e. the gap was 0.002 in.) (Fig. 1). About seven strokes of the plunger gave maximum breakage of cells (71–76%). Homogenization was sometimes continued with a tube and plunger which gave a

Fig. 1. Section of homogenizer: A, stainless-steel conical plunger; B, precision-bore glass tube; C, liver; D, rubber bung.

Fig. 2. Nuclear preparation in medium A. This and subsequent photographs were taken by using a Beck phase-contrast microscope with a 2 mm. objective.

finer gap (0.001 in.). In this case, 76–85% of the cells were finally broken. The medium used for homogenization depended on the purpose of the experiment. Medium A, which gave normal preparations as judged by the criteria outlined above, contained: 0.3M sucrose, 0.065M potassium glycerophosphate, 0.001M-MgCl₂ and 40% glycerol, pH 7.0. The liver tissue was diluted 1 in 5 or 1 in 10 (w/v) for homogenization. Since cells were less easily broken in glycerol-containing solutions, the glycerol was sometimes added after homogenization, by adding to the homogenate an equal volume of the homogenizing medium containing 80% glycerol. All the solutions used were kept at 0–5°.

Centrifuging. For the preparation of nuclei used in most of the small-scale experiments described in this paper, 2 ml. of the homogenate was layered over 8 ml. of medium A. This was possible since the density of rat liver is 1.086 (Anderson, 1955) and the density of medium A was 1.14. The tube (10 ml.) was centrifuged for 10 min. at 730 g in a MSE refrigerated centrifuge ($r_{min.}$ = 12.3 cm.; $r_{max.}$ = 20.3 cm.). After this time the top layer, containing almost entirely mitochondria, was sucked off through a pipette bent through 90° at the tip, and discarded; the middle layer, containing mostly nuclei, was sucked off carefully with a filter pump through a fine glass tube bent through 90° at the tip, and was relayered over 4 ml. of ice-cold medium A, and recentrifuged for 20 min. at 2240 g. The last layering and centrifuging operation was, if necessary, repeated once. This gave a mitochondria-free preparation containing about 50% of the total nuclei of the homogenate, in which there was 8–9% contamination by whole cells or cell fragments (Fig. 2).

Preservation of nuclei by deep-freezing. The homogenates or isolated nuclei were put into Pyrex test tubes (10 mm. $\times$ 75 mm.) closed by rubber bungs. Each tube was sealed by inverting its upper end in acetone-CO₂ at -79° until the thin film of medium round the rubber bung was frozen. It was then lowered into the same freezing mixture and was completely frozen in a few seconds. When it was required to warm up the nuclei or homogenates, the tubes were rotated carefully under the cold-water tap.

EXPERIMENTS AND RESULTS

Expt. 1. Factors affecting granularity of nuclei

As shown by Anderson & Wilbur (1952), ionic strength, the concentration of bivalent cation and the pH are all factors affecting the production of granular nuclei by precipitation of nucleohistone. These three effects are related. Our exploration of this complex interrelation is not yet complete, but the two experiments described below have a bearing on the question.

pH and ionic strength, without added bivalent cation. The relation between pH and the ionic strength required to cause nuclear granularity was investigated by the technique of perfusion of isolated nuclei on the microscope slide. The solutions all contained 0.29M sucrose and 0.02M potassium phosphate buffer at various pH values between 6.0 and 7.2, and KCl at various ionic strengths between 0.03 and 1.03. In each set of experiments a single nucleus was observed and

photographed at a given pH. It was perfused by sucking solutions of increasing ionic strength underneath the coverslip with lens tissue, and photographed at increments of ionic strength of about 0.02. The resulting photographs could be used as points on the figure relating pH and ionic strength, and in this way appearances at different pH values could be conveniently compared. The results showed that with KCl, as also with NaCl, potassium acetate and the potassium salts of ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid, the critical ionic strength above which nuclei are granular (in the absence of added bivalent cation) is about 0.1 at pH 6.8. At pH 6.2 it may be as low as 0.06 and at pH 6.0 as low as 0.03. At all pH values the maximum granularity occurs at $I \approx 0.2$, and as their granularity increases the nuclei may also become irregular and distorted in shape, especially at low pH values. At higher ionic strengths the granularity decreases again, and the normal shape of the nuclei is restored, until at $I = 0.5-1.0$ they are normal in shape and only faintly granular; however, by then they are practically empty, since at this ionic strength both globulins and nucleohistone are soluble. Fig. 3 shows a set of photographs of a nucleus at pH 7.0 between ionic strengths 0.03 and 1.03.

pH and bivalent-cation concentration at constant ionic strength. As might be expected, the bivalent-cation concentration required to produce granular nuclei is dependent on pH at a given ionic strength. For example, in a liver homogenate in sucrose buffered with triethanolamine-HCl ($I = 0.046$)

nuclei were granular below pH 6.25, without the addition of bivalent cation. In addition, the nucleoli showed up well below pH 6.65. This suggested that there was enough bivalent cation present in the homogenate to produce these effects. If the homogenate was first washed twice with 0.3M sucrose and 0.04M potassium EDTA at pH 6.9, no granularity could be produced at pH values down to 6.0 without the addition of bivalent cation, and the nucleoli were invisible over this range. At pH 6.0, 0.001M-CaCl₂ was needed to produce granularity in EDTA-washed nuclei, and at pH 7.0 0.002M-CaCl₂ was needed. Experiments with potassium nitrilotriacetate as a complex-forming agent to buffer the bivalent cation will be published later.

Expt. 2. Factors affecting swelling of nucleus and appearance of nucleoli

The effect of various factors on nuclear size has been investigated by Anderson & Wilbur (1952). They observed that nuclei perfused with sucrose solutions swelled and burst, and suggested that sucrose by itself could be used successfully in isolation media only because of the presence of traces of salts. This suggestion was borne out in the present work, where washing of nuclei with a 0.29M sucrose-0.04M EDTA mixture was used as a method of freeing nuclei from bivalent cations. If these EDTA-washed nuclei were then washed with sucrose, they were not only swollen but surrounded by 'haloes' of nucleohistone dissolving out of the nucleus because of the low ionic strength (Lilienfeld, 1894; Stern, Goldstein, Wagman & Schryver, 1947). It is therefore likely that swelling and clumping together of nuclei in media of low ionic strength (Fig. 4) is the first stage in extraction of nucleohistone from the nucleus, and that halo formation (Fig. 5) is the second stage, which takes place only when all the bivalent cations have been removed. Another effect of low ionic strength and absence of bivalent cations is disappearance of nucleoli, as

Fig. 3. A nucleus photographed at different total ionic strengths at pH 7.0. The appropriate ionic strength is given under each photograph. Composition of medium: 0.02M potassium phosphate buffer, 0.29M sucrose, plus KCl to give varying total ionic strength.

Fig. 4. Swollen and clumped nuclei in 0.29M sucrose.

Fig. 5. Halo around nucleus in 0.29M sucrose, after two washings with 0.29M sucrose plus 0.04M ethylenediaminetetraacetic acid.

mentioned under Expt. 1. Nucleoli appear normal, in the absence of any other ions, at a bivalent-cation concentration above about 0.0005M, at pH 6.9; Ca^{2+} , Mg^{2+} and Ba^{2+} are equally effective. In the absence of bivalent cations the ionic strength has to be higher for preservation of nucleoli; $I \approx 0.08$ gives a normal appearance. It is therefore apparent that the conditions for preservation of nucleoli are similar to those for production of granularity, the limit of bivalent-cation concentration or ionic strength being slightly lower for nucleolar preservation; both conditions are affected in the same way by pH.

Expt. 3. Factors affecting clumping of nuclei and mitochondria

Generally speaking, conditions such as a high ionic strength or bivalent-cation concentration which prevent nuclear clumping encourage mitochondria to clump. This makes separation of nuclei from mitochondria difficult, and since clumping of mitochondria may damage them and allow them to release enzymes harmful to nuclei (see Expt. 6) it should also be avoided for this reason. In addition, the concentration of bivalent cation necessary to prevent nuclear clumping is very near to that which causes nuclear granularity. However, if 60% (v/v) glycerol, 25% acetone or 14% dioxan is added, nuclear clumping can be prevented without addition of bivalent cation. Since these solutions have similar fairly low dielectric constants [60, 67 and 67 at 25° compared with 78 for water; Albright (1937), Critchfield, Gibson & Hall (1953)] their anticlumping effect is possibly due to a change in the ion atmosphere preventing incipient dissociation and accompanying stickiness of nucleohistone. The change in dielectric constant alters the effect of ionic strength

on granularity studied above, but quantitative data on this point are not yet available. It was found that glycerol was the most suitable of these three substances, because acetone and dioxan slowly caused DNA to become non-extractable. Glycerol was harmless to nuclei at all concentrations tried, i.e. up to 70%; 60 and 70% glycerol (cf. Schneider, 1955; Dounce, 1955) also preserved the extractability of nucleohistone from nuclei in a homogenate left for several hours at room temperature, even in the absence of sucrose or glycerophosphate (see Expt. 6), possibly by inhibiting deoxyribonuclease (DNAase). However, the density and viscosity of these high concentrations of glycerol make them impracticable for routine centrifugal sedimentation of nuclei. It was found that 40% glycerol was effective in preventing nuclei from clumping provided that a low concentration of bivalent cation was also present. For example, 40% glycerol and 0.001M- MgCl_2 were used in medium A, and if either substance was omitted clumping of nuclei resulted (Figs. 6, 7). Besides preventing nuclear

Fig. 6. Clumped nuclei in medium A with glycerol omitted.

Fig. 7. Clumped nuclei in medium A with MgCl_2 omitted.

clumping, 40% glycerol prevented contamination of nuclei by mitochondria during centrifugal separation, partly by preventing mitochondrial clumping, and partly by providing, in the layering procedure, a suitable density gradient. A further advantage resulting from the presence of glycerol in the medium was that homogenates and purified nuclei could be deep-frozen at -79° for several weeks without apparent damage (Fig. 8). Dallam (1955) has also described a method for centrifugal separation of nuclei and cytoplasmic granules with high concentrations of glycerol.

Expt. 4. Factors affecting bleb formation on nuclei

Anderson (1953a) showed that the formation of blebs or bubbles on the surface of nuclei in distilled water or various concentrations of salts could be prevented by the presence of concentrations of sucrose of 0.05M and above. The fact that this bleb formation occurred in isolated nuclei suggests that it was a non-enzymic phenomenon, or at any rate was not due to the liberation of degradative enzymes from mitochondria. However, Anderson (1953b) has shown also that bleb formation is increased in nuclei which have previously been treated with DNAase, and may then occur even in isotonic media. In the present experiments blebs were formed on nuclei left in media of quite high ionic strength in the presence of mitochondria, provided that neither sucrose nor glycerophosphate was present (Fig. 9). They were often accompanied by a loss of density of the nucleus, which has been shown by Anderson (1953b) to be a result of DNAase action (see Expt. 6).

Fig. 8. Nuclei in medium A, after deep-freezing at -79° for 3 weeks.

Fig. 9. Bleb formation by nucleus in medium containing 0.06M-NaCl, 0.005M potassium phosphate buffer (pH 7.0) and 40% glycerol.

Expt. 5. Factors affecting extractability of DNA from nuclei

If the nucleoprotein of the nucleus has been degraded by the action of mitochondrial enzymes, or by non-enzymic means, it will no longer be extractable from the nucleus by M-NaCl or -KCl as a viscous gel forming a 'halo' round the nucleus. This appears to be a very sensitive test for degradation, more so than the macroscopic test of gel formation (Dounce, 1955; Dounce & Monty, 1955) and one which is easily applied both to free nuclei if there are surrounding mitochondria to show the halo up, and to nuclei still inside cells, which behave in the same way as free nuclei (Fig. 10). The halo round free nuclei can also be shown up, in the absence of mitochondria, by methyl-violet staining (Fig. 19). In this work the test of halo formation was used to check the suitability of various suspension media for nuclei. The following solutions preserved the extractability of nucleohistone in the presence of mitochondria at pH 6.9 for some hours.

Sucrose; glycerophosphate. Sucrose or glycerophosphate or both (see Expt. 6), probably acting by preventing liberation of hydrolytic enzymes from mitochondria.

Glycerol. Glycerol (70%, w/v) (see Expt. 3), probably acting by inhibiting hydrolytic enzymes.

0.2M-NaCl or 0.2M-KCl. These were tried because of the finding by Webb (1953) that 0.2M-NaCl inhibited the DNAases of calf thymus and leukaemic mouse tissue. Both of course made the nuclei granular, but this granularity was reversible on washing, and good haloes were subsequently formed on addition of M-NaCl.

0.06M-MgSO₄. This was tried for the same reason, and worked about as well as NaCl and KCl; it also made nuclei reversibly granular.

Fig. 10. Halo around nucleus inside cell, formed by suspending freshly prepared homogenate in medium A in M-NaCl.

0.01M-ZnSO₄. This was also mentioned by Webb as an inhibitor of DNAases. It was rather less effective than the other inhibitors.

These findings suggest that in the absence of sucrose or glycerophosphate any substance which inhibits DNAase without at the same time denaturing nucleohistone will protect the nucleohistone while the mitochondria are being removed. However, 70 % glycerol is troublesome as a medium for centrifugal separation, and 0.2M-NaCl or -KCl would be expected to extract globulins from nuclei; these have been shown (Kirkham & Thomas, 1953) to constitute one-third to one-half of the dry weight of the nuclei. It is also not certain that nuclei which have been made granular by any means are completely undamaged, even if the granularity is reversible. It is therefore probable that the simplest and most convenient method of preserving nucleohistone is to keep the mitochondria intact by means of sucrose or glycerophosphate while they are being removed.

Various substances besides m-NaCl or m-KCl will extract DNA from intact nuclei, and will produce haloes round the nucleus. These include heparin (Anderson & Wilbur, 1951), 0.05M sodium hexametaphosphate, 0.03M potassium triphosphate (K₃P₃O₁₀), 0.03M adenosine triphosphate, 0.01M potassium polymethacrylate, 0.06M sodium barbital buffer, 0.3M triethanolamine-HCl buffer, 0.3M tetraethylammonium chloride and 0.2M tetramethylammonium chloride. (The concentrations given are the lowest needed to produce clear and definite haloes at pH 6.9.) The first five substances probably act by displacing nucleic acid from histone.

If a solution of histone itself (calf-thymus histone, supplied by Dr P. W. Kent) is added to a homogenate in sucrose, a characteristic type of peripheral and irregular granularity of the nucleus appears (Fig. 11). This happens at quite low concentrations of histone (0.14 %, containing about 0.003M amino groups). It is probably due to precipitation of the histone with nucleic acid on the nuclear membrane, and may be related to the Liesegang-ring phenomenon, depending on the counter-diffusion of DNA and histone. This was investigated in experiments in which 0.04M potassium triphosphate was added to a homogenate, together with varying amounts of histone. When the histone was equivalent to 0.003M amino groups, haloes were formed as usual by the triphosphate, but there was no peripheral granularity. In this case presumably there was no region in which the counter-diffusion of DNA and histone allowed the solubility product of nucleohistone to be reached. When the equivalent concentration of histone was 0.015M, both haloes and peripheral granularity appeared, suggesting that the solubility product of

nucleohistone was reached at the nuclear membrane but not outside it, although some DNA was still diffusing through. If the histone concentration was increased still further, there was peripheral granularity but no halo formation, presumably because of complete precipitation of DNA by histone at the membrane. If 0.2 % protamine sulphate (British Drug Houses Ltd.) was added to a nuclear preparation by perfusion under the coverslip, the nuclei shrank and became extremely refractive. A fine granularity appeared all through the nucleus, which later condensed into large lumps (Fig. 12). Since protamine has a smaller molecular weight than histone, it diffuses further into the nucleus before precipitating with DNA.

Fig. 11. Peripheral granularity of nuclei (inside a binucleate cell), produced by addition of 0.14 % calf-thymus histone to homogenate in sucrose plus potassium phosphate buffer (pH 6.8).

Fig. 12. Internal granularity of nuclei, produced by perfusion on a slide with 0.2 % protamine sulphate. (Nuclei were prepared in 0.115M potassium glycerophosphate, 40 % glycerol, 0.3M sucrose and 0.001M-MgCl₂.)

Expt. 6. Further studies of media containing potassium glycerophosphate

The work of Appelmans & Duve (1955) on 'osmotic activation' of mitochondria, and its prevention by sucrose and potassium glycerophosphate, led to the hypothesis that sucrose protected nuclei in a homogenate from enzymic bleb formation and emptying by preventing liberation of harmful enzymes, probably DNAase from mitochondria. Potassium glycerophosphate was therefore tried as a whole or partial substitute for sucrose, and was found to be even more effective than iso-osmolar concentrations of sucrose in protecting nuclei (Philpot & Stanier, 1955). For example, 0.091M or 0.115M potassium glycerophosphate (osmotically equivalent to 0.24M and 0.3M sucrose respectively) preserved nuclei equally well, whether or not sucrose was also present, but the higher concentration was the more effective; concentrations higher than 0.115M were no better. 0.065M Potassium glycerophosphate (osmotically equivalent to 0.17M sucrose) preserved nuclei better than 0.3M sucrose, but the combination of 0.065M potassium glycerophosphate and 0.3M sucrose proved better than these concentrations of either substance alone, so here the effect seemed to be additive.

Effect of amount of mitochondrial contamination

The effect of mitochondrial contamination in hastening emptying of nuclei was shown by isolating nuclei in the complete protective medium (0.115M potassium glycerophosphate, 0.3M sucrose, 40% glycerol, pH 7.0), and then resuspending them, with or without the addition of mitochondria, in either the same medium or the same medium without potassium glycerophosphate; the latter medium contained 0.01M potassium phosphate buffer, pH 7.0. All four preparations were left at room temperature for 25 hr., and then photographed.

Figs. 13 and 14 show nuclei from preparations to which mitochondria had not been added. The nuclei from the medium containing potassium glycerophosphate are full (Fig. 13), and those from the medium without potassium glycerophosphate almost equally so (Fig. 14). Figs. 15 and 16 show nuclei from preparations in which mitochondria were present. When potassium glycerophosphate was present the nuclei again remained full (Fig. 15), but when it was absent they became empty (Fig. 16), even though sucrose was present.

The fact that the protective action of potassium glycerophosphate on nuclei is seen only or mainly in the presence of mitochondria supports the theory that it acts by protecting nuclei from mitochondrial enzymes. However, nuclei among mito-

chondria eventually become empty even in the presence of potassium glycerophosphate, and the number of mitochondria present will determine how soon this happens. For example, 0.115M potassium glycerophosphate (with or without sucrose) prevented nuclei from emptying for 46 hr. at room temperature in the presence of mitochondria, but for up to 100 hr. in a preparation which had been partially freed of mitochondria by two centrifugings. The nuclei retained their 'full' appearance and their ability to form haloes on addition of m-NaCl (Figs. 17-19). The only difference seen in the preparations without sucrose was possibly a greater tendency to bleb formation; even in the presence of sucrose there was some bleb formation after 20 hr. at room temperature.

Fig. 13

Fig. 14

Fig. 15

Fig. 16

Figs. 13-16. Nuclei after 25 hr. at room temperature in: Fig. 13: complete medium (0.115M potassium glycerophosphate, 40% glycerol, 0.3M sucrose) without mitochondria; Fig. 14: without potassium glycerophosphate medium and without mitochondria; Fig. 15: complete medium with mitochondria; Fig. 16: medium without potassium glycerophosphate and with mitochondria.

Fig. 17. Nuclei after standing at room temperature for 73 hr. in medium containing 0.115M potassium glycerophosphate, 40% glycerol and 0.0006M-MgCl₂.

Fig. 18. Haloes around nuclei inside cells, formed by suspending in M-NaCl a homogenate which had stood at room temperature for 67 hr. in 0.115M potassium glycerophosphate, 40% glycerol, 0.3M sucrose and 0.0002M-MgCl₂.

Fig. 19. Methyl-violet-stained halo around free nucleus. Halo was formed by suspending in M-NaCl a nuclear preparation which had stood at room temperature for 96 hr. in 0.115M potassium glycerophosphate, 40% glycerol, 0.3M sucrose and 0.001M-MgCl₂; aqueous methyl violet was run in under the coverslip.

Comparison of potassium glycerophosphate with potassium phosphate and other buffers

To investigate the possibility that potassium glycerophosphate was preserving nuclei simply by acting as a pH buffer, its effectiveness was compared with that of lutidine, barbitone and triethanolamine buffers at pH 6.9: 0.3M sucrose and 40% glycerol were the other components of the system. With all three buffers nuclei started to look empty and DNA became non-extractable in a viscous form after the homogenate had stood for an hour or two at room temperature. The possibility was tested that potassium glycerophosphate was working by virtue of its osmolarity or its ionic strength or both. Accordingly, a system was tested which contained K₂SO₄, lutidine and sucrose and had the same osmolarity, ionic strength and buffering power as 0.065M potassium glycerophosphate and 0.3M sucrose. It proved to be no more effective than the simple buffer systems. On the other hand, potassium phosphate buffer (pH 6.9), used at concentrations much higher than that needed for pH buffering, appeared at first sight to be almost as effective as potassium glycerophosphate, especially when sucrose was present; the concentration needed for full effect (0.125M) was a little higher than that of potassium glycerophosphate. However, long-term experiments showed that it was considerably less effective, and was much more sensitive than potassium glycerophosphate to the absence of sucrose. In a typical experiment, liver homogenates in the solutions under test were washed by centrifuging for 15 min. at 25 000 g, followed by resuspension in the same solution. They were then allowed to stand at room temperature, and were examined at intervals. After 46 hr. the preparation in 0.125M potassium phosphate and 40% glycerol contained few full nuclei, many blebs, and only a few nuclei which formed haloes on suspension in M-NaCl. If 0.3M sucrose was present as well, there were more full nuclei and fewer blebs after this time, and haloes were more consistently formed. The preparation in 0.115M potassium glycerophosphate and 40% glycerol contained full nuclei, some of which had formed blebs, and good haloes were produced on addition of M-NaCl. The preparation containing 0.3M sucrose in addition showed no improvement on this, except that only very occasional blebs were seen.

Effect of glycerol on emptying

In all these solutions, 40% glycerol was used to prevent clumping of nuclei, and in addition it appeared to have a convenient bacteriostatic action. A previous experiment had shown that the ability of a preparation to form 'haloes' is not altered by the presence of 40% glycerol.

Effect of MgCl₂ and CaCl₂ on emptying

0.115M Potassium glycerophosphate apparently provides a sufficiently high ionic strength to prevent nuclei from clumping, provided that 40% glycerol is also present. In the experiment described above, in which the nuclei were unclumped after centrifuging in 0.115M potassium glycerophosphate and 40% glycerol, MgCl₂ was present at various concentrations between zero and 0.04M to test its effect on nuclear emptying. The concentration of MgCl₂ appeared to have little effect on the preservation of the nuclei, though there was a suggestion that the nuclei, which started by being very granular (at MgCl₂ concentrations of 0.005M and above), formed less consistently good haloes later on, even though this granularity gradually decreased or disappeared. On the other hand, 0.001M-CaCl₂ was shown to hasten the emptying of nuclei in a homogenate; this effect was accompanied by a characteristic 'fluffy' appearance of the mitochondria, even in the presence of 0.3M sucrose.

The experiments described in Expt. 6 are consistent with the theory that sucrose and potassium glycerophosphate protect nuclei by preventing damage to mitochondria and their consequent liberation of harmful enzymes. This enzymic explanation was given additional support by the demonstration of Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) that DNAase, ribonuclease and cathepsin are among the enzymes associated with 'lysosomes', the mitochondrial granules rich in hydrolytic enzymes, from which they are liberated by 'osmotic activation'. The present results, and the experiments on inhibitors mentioned under Expt. 5 are consistent with the enzyme concerned being DNAase; although Dounce, Witter, Monty, Pate & Cottone (1955) and Dounce & Monty (1955) who have reached the same conclusion about the importance of protecting mitochondria if the gel-forming power of nuclei is to be preserved, consider that there is some evidence against this.

DISCUSSION

Some of the rules for the design of a nuclear medium given above, which are largely empirical, can be explained in terms of the concept of the nucleus that has emerged from the work of many investigators but which is still to some extent controversial. One form of this concept may be stated as follows.

The normal resting nucleus is an optically homogeneous nucleohistone gel enclosed in a coarsely permeable membrane and containing a few denser

structures, the nucleoli. Its submicroscopic interstices contain various 'euglobulins' (i.e. proteins insoluble at low ionic strength), some of them enzymes, which are in gel form but which can be dissolved by ionic strengths above about 0.1, or higher if the dielectric constant is lowered by glycerol. The cell sap probably penetrates freely through the nuclear membrane, and its components may reach into the interstices of the gel to an extent probably dependent on their molecular weights. Whether this penetrating cell sap is part of the nucleus is a purely linguistic question which is nevertheless of importance because it probably underlies the controversy between the advocates of wet and dry methods of isolating nuclei. The nucleohistone gel has a net negative charge (cf. Philpot & Stanier, 1954) which would be expected to give it electro-osmotic properties. It therefore swells and even dissolves as the ionic strength is lowered (Stern *et al.* 1947), provided that bivalent cations are absent, while at higher ionic strength (about 0.14) it precipitates, making the nucleus granular, and at still higher ionic strength it dissolves by dissociation of the histone from the nucleic acid (Mirsky & Pollister, 1946). Possibly also the spare negative charges of the DNA can form dissociable salt-linkages with the non-histone protein, and may be partly or wholly responsible for the euglobulin properties of the latter (cf. Dounce, 1952). The granularity produced by change of ionic strength, pH or bivalent-ion concentration, or the visibly different type produced by histone, may be related to the condensation of chromosomes at mitosis, but is less regularly arranged. Possibly a better resemblance could be produced by slower precipitation.

There is thus a wide choice of media that can keep the nucleohistone and the globulins in their insoluble-gel form; but it is also necessary during isolation of nuclei to keep the mitochondria or rather the 'lysosomes' (Duve *et al.* 1955) in good condition so that they do not liberate lytic enzymes such as nucleases and cathepsin, which can cause rapid emptying of nuclei and also bleb formation (Anderson, 1953b). This probably accounts partly or wholly for the beneficial effect of glycerophosphate and sucrose. We suspect that glycerophosphate acts mainly by preventing some degradation inside the lysosome, leading to low-molecular breakdown products with a high intrasomal osmotic pressure, which can be balanced by external sucrose to prevent bursting. If this is true, metabolically normal lysosomes would need less sucrose, which may explain the absence of sucrose or of any obvious substitute in natural cell sap, and which may also explain why glycerophosphate can prevent nuclear emptying at lower osmolality than is needed with sucrose.

SUMMARY

1. For isolation of rat-liver nuclei a medium is recommended having this composition: 0.3M sucrose, 0.065M potassium glycerophosphate, 0.001M magnesium chloride, 40% glycerol; pH 7.0.

2. Phase-contrast photographs are shown of nuclei in this medium, and in modifications of it causing various abnormal appearances.

3. Nuclei in the recommended medium retain their normal appearance for at least 6 hr. at room temperature, after which they are still able to form haloes of soluble viscous nucleic acid when transferred to molar sodium chloride.

4. If the glycerophosphate concentration is increased to 0.115M, with or without magnesium chloride, nuclei retain their normal appearance for 100 hr. at room temperature, apart from slight clumping; but the higher ionic strength of this medium may sometimes be a disadvantage.

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Cerebral Diphosphoinositide Breakdown: Activation, Complexity and Distribution in Animal (mainly Nervous) Tissues

By R. RODNIGHT

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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Suspensions of guinea-pig brain rapidly attack the 'inositol phosphatide' fraction of ox-brain kephalin, liberating into the medium acid-soluble inositol and phosphate (Sloane-Stanley, 1953). Presumably cerebral tissue contains enzymes catalysing the breakdown of the diphosphoinositide (DPI), shown by Folch (1942) to be an important constituent of brain kephalin. The interesting feature of this reaction is its rate, which is outstandingly higher than the rates previously reported for the *in vitro* hydrolysis of other brain lipids (Sloane-Stanley, 1952). On a molar basis it is comparable to the rate of oxygen uptake by cerebral preparations.

The present work is mainly concerned with the distribution of the enzyme system and its sensitivity to certain cations and drugs. For most experiments a saline suspension of the 'inositol phosphatide' fraction of kephalin has been used as substrate, but preliminary observations on the breakdown of the water-soluble potassium salt of cerebral DPI (Folch, 1949a) are also included.

EXPERIMENTAL

Enzyme activity was studied in two ways. (a) By following acid formation manometrically in bicarbonate-buffered saline solutions (Sloane-Stanley, 1953). The rate of CO₂