A Comparison of Cell Nuclei Isolated from Rabbit Tissues by Aqueous and Non-aqueous Procedures

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In a previous paper (Smellie, Humphrey, Kay & Davidson, 1955) we have described experiments on the incorporation of radioactive phosphorus (^{32}P) into the nucleic acids of cell nuclei prepared from several different rabbit tissues by the usual citric acid technique. In such nuclei most of the acid-soluble constituents, and probably also some of the protein, have been lost during the isolation process. This loss can be avoided by using nuclei isolated in non-aqueous solvents by the original procedure of Behrens (1932, 1938), or by the more recent modifications of Dounce, Tishkoff, Barnett & Freer (1950) and of Allfrey, Stern, Mirsky & Saetren (1952). Experiments with such nuclei are described in this paper.

EXPERIMENTAL

Isolation of nuclei from non-aqueous solvents (non-aqueous nuclei, NAN). Young albino rabbits were used in these experiments and the following modification of the procedure of Allfrey *et al.* (1952) was employed. It is outlined in Fig. 1.

Immediately after the animal had been killed, the tissues required (appendix, thymus, small intestinal mucosa, bone marrow and lung) were excised. Whenever possible the tissue was immediately cut into a mash with scissors, transferred to a wide-mouthed flask with a ground-glass neck, frozen in a solid CO_2 -ethanol mixture and dried from the frozen state. Tissues that could not be immediately processed were packed in solid CO_2 and stored in a deep-freeze cabinet.

The thoroughly dried tissue was weighed and ground to a powder in a mortar. Since the weight of dry powder was usually not more than 5 g., it was found practicable to disintegrate the tissue with the aid of a plastic homogenizer of the Potter-Elvehjem type rather than in a ball mill as used by previous authors.

The powder (5 g.) was disintegrated with about 30 ml. of light petroleum (b.p. $40-60^\circ$) until the cells were completely ruptured, as revealed by frequent microscopical examinations. The dispersion was filtered through four layers of muslin and the fibrous residue discarded. The filtrate was centrifuged with three successive lots of light petroleum (30-40 ml.) at 1500 g for 3 min., the supernatant fluid being discarded. The sediment was suspended in a mixture of cyclohexane- CCl_4 (1:1, v/v) and centrifuged at 1500 g

for 5 min., the supernatant fluid being discarded. This process was repeated twice. The sediment was then mixed with about 20 ml. of cyclohexane- CCl_4 and ground in the homogenizer again, if necessary. The suspension was adjusted to sp.gr. 1.320 by the addition of CCl_4 to the mixture, to make a total volume of approximately 40 ml.,

Fig. 1. Scheme of isolation of nuclei in non-aqueous media.

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which was then centrifuged for 30 min. at 1500 *g*. The supernatant fluid was decanted, the sediment mixed with about 20 ml. *cyclohexane*- CCl_4 and the sp.gr. of the mixture adjusted to 1.335 with additions of CCl_4 and *cyclohexane*. The mixture was centrifuged at 1500 *g* for 30 min. The supernatant fluid was discarded and the sediment mixed with about 20 ml. of *cyclohexane*- CCl_4 (1:1, v/v).

At this stage, if the disintegration had been adequate, the nuclei were essentially clean and free from obvious cytoplasmic contamination. The suspension was finally centrifuged in the *cyclohexane*- CCl_4 mixture for 10 min. at 1500 *g*. The supernatant fluid was discarded and the sediment mixed with about 40 ml. of light petroleum and centrifuged at 1500 *g* for 5 min. The light petroleum was decanted and the nuclear residue air-dried and stored in a desiccator.

A few preparations were made of nuclei from rabbit-embryo liver, the steps in the procedure being identical with those described above, but further stages were required to remove the finely divided cytoplasmic material. The nuclear sediment obtained from the mixture at sp.gr. 1.335 was resuspended in *cyclohexane*- CCl_4 and the sp.gr. adjusted to 1.350 with CCl_4 . The mixture was centrifuged at 1500 *g* for 30 min. and the supernatant fluid discarded. The sediment (80–95% nuclei) was mixed with *cyclohexane*- CCl_4 and the sp.gr. adjusted to 1.370. The mixture (about 40 ml.) was centrifuged at 1500 *g* for 30 min. The supernatant fluid was discarded and the nuclear sediment mixed with *cyclohexane*- CCl_4 and centrifuged at 1500 *g* for 5 min. The supernatant fluid was discarded and the nuclear sediment mixed with light petroleum and centrifuged at 1500 *g* for 5 min. The final sediment was air-dried and stored.

All manipulations were carried out at room temperature.

Staining and microscopical procedures. All stages of the isolation procedure were followed by careful microscopic observations of the preparations. Smears were made on microscope slides, dried and stained with crystal violet (0.1%) in 0.1 *M* citric acid, with pyronin-methyl green or with aceto-orcein-fast green. The last stain is particularly useful (Kurnick & Ris, 1948), since nuclei are stained a reddish colour, whereas cytoplasmic and intracellular material appears greenish blue. Consequently any tags of cytoplasmic material adhering to the nuclei are easily noticed.

Isolation of citric acid nuclei (CN). This was carried out as described previously (Smellie *et al.* 1955).

Non-aqueous nuclei extracted with citric acid (ENAN). In certain experiments on the effect of citric acid in removing nuclear constituents, portions of preparations of NAN from thymus, appendix and intestinal mucosa were ground with ice-cold 0.05 *M* citric acid in a Potter-Elvehjem homogenizer and centrifuged for 10 min. at 666 *g*. The supernatant fluid was preserved. The sedimented nuclei were washed twice with 0.01 *M* citric acid and centrifuged at 375 *g* for 5 min.

In some experiments the pH of the citric acid used for extraction was adjusted to 3.5 in order to make the conditions comparable with those obtaining in the preparation of CN from whole tissue.

The citric acid extract (CE), consisting of combined supernatant and washings, was centrifuged at 666 *g* for 15 min. to remove particulate matter. The supernatant fluid was treated with 0.5 vol. of 30% (w/v) trichloroacetic acid (TCA) and the precipitate, which was abundant, washed twice with 10% (w/v) TCA, extracted with lipid solvents and treated as described below.

Chemical manipulations. Samples of NAN and CN were extracted with 10% (w/v) TCA and lipid solvents as described by Smellie *et al.* (1955). The dry residue was incubated with 0.5 *N*-KOH according to the modification of the Schmidt & Thannhauser (1945) method that we have previously employed.

The digest was acidified to pH 1 with 10 *N*- HClO_4 , the precipitated deoxyribonucleic acid (DNA) and protein centrifuged down and washed with 0.5 *N*- HClO_4 , the washings being added to the ribonucleotide fraction in the supernatant. P determinations were carried out on each fraction in order to give the total ribonucleic acid (RNA) and DNA contents of the preparations.

In those experiments in which ^{32}P was used, the general procedure of Smellie *et al.* (1955) was employed, the nuclear ribonucleotides being separated by ionophoresis on paper and the DNA being extracted with sodium dodecyl sulphate from the nucleoprotein precipitate in the Schmidt-Thannhauser separation.

All P estimations were carried out by a micro-modification of the method of Allen (1940). Pentose was estimated by Meijbaum's (1939) method, as described by Davidson & Waymouth (1944), with ribose as standard, and deoxypentose was estimated as described by Davidson & Waymouth (1944), with purified DNA as standard.

Isotopic techniques. In some experiments the animals received an intramuscular injection of 1 mc of ^{32}P as inorganic phosphate, 2 or 18 hr. before killing. The handling of labelled material was carried out as previously described (Smellie *et al.* 1955). Where results are expressed as relative specific activity (r.s.a.), the value for blood inorganic phosphate 2 hr. after ^{32}P administration has been taken as 10 000.

RESULTS

In most of the preparations of NAN the amount of contamination from whole cells and cytoplasmic debris appeared to be negligible on microscopical inspection, although, in trial preparations from kidney, and especially from adult liver, great difficulty was experienced in removing much of the finely divided cytoplasmic material and partially disintegrated cells. Plate 1 shows the progress of

Plate 1. Photomicrographs showing stages in the preparation of non-aqueous nuclei. Kodak Ektachrome film. Magnification $\times 520$. (This was the original magnification of the photographs; they have been reduced by about one-third for the purpose of block-making.) Aceto-orcein-fast green stain. A, Section of rabbit thymus showing the high proportion of non-nuclear material; B, section of rabbit appendix; C, partially purified preparation of nuclei from rabbit intestinal mucosa (the contaminating cytoplasmic debris stains green); D, preparation of appendix nuclei (a green cytoplasmic tag is visible on one nucleus); E, purified preparation of intestinal-mucosa nuclei; F, purified preparation of thymus nuclei.

the isolation of NAN from appendix, thymus and intestinal mucosa. A and B are sections of the original appendix and thymus tissue illustrating the initial relative proportions of non-nuclear and nuclear material; C shows a preparation of intestinal-mucosa nuclei at an early stage in the isolation process, with a large amount of cytoplasmic debris still in evidence; D shows a preparation of nuclei almost at the final stage; E and F illustrate final preparations of NAN. From these illustrations it is clear that the final preparations are completely free from obvious contamination with cytoplasmic matter. Our main criterion of the purity of our preparations is indeed the appearance of stained nuclei on microscopical examination.

obtain a rough estimate of the protein content by difference. The amount of protein as shown in Table 1 varies between 79 and 92% of the dry weight.

The corresponding data for a series of preparations of CN from appendix, intestinal mucosa and thymus are also shown in Table 1. Although the figures for the percentage of DNA-P are much higher, those for RNA-P are appreciably lower, with the result that the ratios RNA-P/DNA-P are much lower for CN than for NAN. The higher percentage of total nucleic acid in CN indicates a correspondingly reduced protein content. These observations are consistent with the view, which is discussed below, that citric acid removes appreci-

Table 1. *Composition of cell nuclei isolated from rabbit tissues by the non-aqueous procedure (NAN) and by the citric acid method (CN)*

Tissue	DNA-P % dry weight*		RNA-P % dry weight*		RNA-P/DNA-P		Protein approx. % dry wt. (by diff.)†		Protein/ DNA-P†	
	NAN	CN	NAN	CN	NAN	CN	NAN	CN	NAN	CN
Appendix	1.01 (13)	2.5 (5)	0.48 (13)	0.32 (5)	0.48	0.13	84	71	8.1	2.8
Intestinal mucosa	0.64 (9)	2.3 (5)	0.45 (9)	0.38 (5)	0.71	0.17	88	72	13.5	3.1
Thymus	1.68 (10)	3.1 (5)	0.35 (10)	0.24 (5)	0.21	0.08	79	66	4.6	2.1
Bone marrow	0.67 (6)	—	0.30 (6)	—	0.45	—	89	—	13.0	—
Lung	0.45 (12)	—	0.27 (12)	—	0.60	—	92	—	20.0	—
Embryo liver	0.38 (3)	—	0.37 (3)	—	0.975	—	91	—	22.2	—

* Numbers in parentheses refer to number of preparations examined.

† In making these calculations it has been assumed that the P content of the Na salt of RNA is 7.8% and of the Na salt of DNA is 9.3% (Kay & Dounce, 1953).

Table 2. *Relative specific activities of the DNA and RNA of citric nuclei (CN) and non-aqueous nuclei (NAN) isolated from rabbit appendix and from small intestinal mucosa of rabbits which had received 1 mc of ³²P*

Nucleic acid	Time after administration of ³² P (hr.)	Appendix			Intestinal mucosa			Thymus		
		CN	NAN	NAN/CN	CN	NAN	NAN/CN	CN	NAN	NAN/CN
DNA	2	1373	1380	1.0	258	373	1.4	434	417	1.0
RNA	2	3765	1665	0.4	959	592	0.6	2229	713	0.3
DNA	18	2692	2705	1.0	585	602	1.0	—	—	—
RNA	18	2625	2860	1.1	1196	1645	1.4	—	—	—

The results of chemical analyses of several different preparations of NAN from various tissues are listed in Table 1, which shows that the content of DNA phosphorus (DNA-P), expressed as a percentage of the dry weight, varies considerably according to the tissue of origin, the highest value being found in thymus. The RNA phosphorus (RNA-P) content calculated from the total P in the ribonucleotide fraction varies within a narrower range (0.27–0.48%). In all cases the RNA-P content is less than the DNA-P content.

Since protein and nucleic acids are the only abundant constituents of nuclei, it is possible to

able amounts of both protein and RNA from cell nuclei.

Comparison of the relative specific activities of the nucleic acids in CN and NAN shows that the activities of the DNA's isolated from CN and NAN are closely similar in all tissues examined except intestinal mucosa. The results of one experiment are shown in Table 2, from which it is clear that the DNA of appendix and thymus has the same specific activity whether it is isolated from CN or NAN; in intestinal mucosa, however, the DNA from NAN has a higher activity 2 hr. after ³²P administration than has that from CN, the ratio

being about 1.4:1. This anomalous behaviour of intestinal-mucosa DNA has been repeatedly observed, the values found in a series of experiments being 1.4, 1.4, 1.6, 1.8, 1.4, 1.6 and 1.5 at 2 hr. after administration of ^{32}P , whereas the value for appendix and thymus DNA in a similar series was 1. (The same effect is seen also in Table 5.) At 18 hr. after ^{32}P administration the difference between the relative specific activities of the DNA from CN and NAN of intestinal mucosa is not found.

To demonstrate that the difference in the specific activities of the DNA's from the two types of nuclei was not due to contamination, specimens of DNA from CN and NAN prepared from the intestinal mucosa of the same animal were degraded enzymically to the constituent deoxyribonucleotides by the method described by Smellie *et al.* (1955). The results of one experiment given in Table 3 show that the difference persists among the mononucleotides.

Table 3. *Relative specific activities of individual deoxyribonucleotides of the DNA's prepared from citric nuclei (CN) and non-aqueous nuclei (NAN) of rabbit small-intestinal mucosa. ^{32}P was injected 2 hr. before killing*

	CN	NAN	NAN/CN
Deoxyadenylic acid	261	530	2.01
Deoxyguanylic acid	366	520	1.42
Deoxycytidylic acid	245	455	1.86
Thymidylic acid	366	545	1.49

Table 4. *Ratio of RNA-P to DNA-P in citric acid tissue homogenates in citric nuclei (CN) in light-petroleum homogenates, in non-aqueous nuclei (NAN) and in preparations of NAN at an intermediate stage in the course of isolation*

These figures represent the results of a single experiment; hence the values quoted show minor differences from the figures quoted in Table 1, which are mean values.

Tissue	Citric acid homogenate	CN	Light-petroleum homogenate	Intermediate stage in isolation of NAN	NAN
Appendix	0.45	0.17	0.49	0.39	0.38
Bone marrow	0.57	0.09	0.51	0.43	0.34
Intestinal mucosa	0.92	0.25	0.90	0.66	0.51
Thymus	0.29	0.06	0.30	0.22	0.21

The specific activity of the RNA obtained from CN was invariably found to be higher than that obtained from NAN in all tissues examined at 2 hr. after ^{32}P administration. This difference was not found at 18 hr. (Table 2).

These differences between CN and NAN might be explained on the assumption that citric acid treatment of nuclei results in the removal of appreciable amounts of phosphorus compounds, as well as of protein, and experiments were therefore carried out to determine the ratio RNA-P/DNA-P in the same specimen of the same tissue in homo-

genates in citric acid, in CN, in homogenates of frozen dried tissue in light petroleum, in NAN and in material at an arbitrary intermediate stage in the preparation of NAN. The results of such an experiment are given in Table 4, which shows a sharp drop in the ratio RNA-P/DNA-P when CN are isolated from the homogenate. During the preparation of NAN the ratio also falls, as might be expected, owing to the removal of cytoplasmic material, but the final values are invariably higher than those found with CN from the same sample of tissue from the same animal. These results would be consistent with the view that citric acid extracts some RNA from the nucleus. It should also be recorded that thymus nuclei prepared in diethylene glycol by a method similar to that employed by Tyner, Heidelberger & LePage (1953) gave an RNA-P/DNA-P value of 0.21, corresponding to the value found for NAN rather than for CN.

We have also carried out experiments to determine the nature and activity of compounds extracted from NAN by citric acid and of those remaining. The materials extracted will obviously include the acid-soluble constituents of NAN, which must to a large extent be leached out during the process of isolation of CN.

When specimens of NAN were treated with citric acid under conditions similar to those obtaining in the preparation of CN, the citric acid extracts plus washings (CE) were found to contain appreciable amounts of both P and N. When such extracts of NAN from appendix, intestinal mucosa

and thymus were treated with TCA, abundant precipitates consisting mainly of protein appeared. These precipitates accounted in one experiment for 23, 18 and 14 % respectively of the dry weights of the NAN from appendix, intestinal mucosa and thymus.

The supernatant fluids from such precipitates showed negligible amounts of materials reacting as deoxypentose. They did, however, contain appreciable amounts of phosphorus and of pentose-reacting substances, mainly simple nucleotides. Indeed, we have shown in other experiments, to be

published later, that among the acid-extractable constituents of NAN are ribonucleotides of adenine, guanine, hypoxanthine, uracil and, in smaller amounts, cytosine.

When pentose estimations were carried out on TCA extracts of NAN and of CN, it was found that 85–90% of the acid-soluble pentose-reacting materials in the NAN had been removed by citric acid. Similar estimations on TCA extracts of the residual NAN after citric acid extraction (ENAN) confirmed that most of the pentose derivatives had been extracted. These pentose derivatives are mainly purine ribosides or ribonucleotides.

When the precipitates obtained by the addition of TCA to CE were submitted to the Schmidt–Thannhauser procedure only very slight traces of DNA were found, but such precipitates contained almost as much RNA as was left behind in the ENAN. It would appear therefore that citric acid removes appreciable amounts of RNA from NAN. On the other hand, the conditions of extraction of NAN with citric acid are not strictly comparable with those obtaining in the treatment of a whole tissue homogenate with citric acid, since the cytoplasmic proteins can exert an appreciable buffering effect. Accordingly, in one experiment the pH of the citric acid used to extract NAN was adjusted to 3.5 so as to obtain conditions more like those found in the treatment of whole tissue homogenates. Although in such circumstances the amount of RNA extracted was somewhat reduced it was still appreciable.

Since the specific activity of the RNA was usually higher in citric nuclei than in non-aqueous nuclei it was of some interest to determine the activity of the RNA in the citric acid extract. In one such experiment on thymus nuclei the relative specific activity in the RNA from CN was 2229, whereas that in NAN was 713. When the NAN were extracted with citric acid the relative specific activity of the RNA extracted was 241, whereas that of the RNA remaining in the ENAN had risen to 900. These observations suggest that the RNA in NAN is not homogeneous and that citric acid preferentially removes a fraction of lower activity.

We have already mentioned that citric acid does not extract DNA from NAN; consequently it might reasonably be expected that the specific activity of the DNA would be of the same order in NAN as in ENAN. That this is in fact the case is demonstrated in Table 5 for appendix, intestinal mucosa and thymus. In intestinal mucosa the difference in the activity of the DNA isolated from CN and from NAN is still found in ENAN after citric acid extraction and is not therefore due to the removal by citric acid of material of particularly high activity.

When DNA is extracted by sodium dodecyl sulphate from the nucleoprotein precipitate obtained in the Schmidt–Thannhauser separation, appreciable amounts of P still remain in the protein residue and the relative specific activities of this residual P are usually of the same order as those for DNA. This residual P is always considerably larger in amount in NAN than in CN and ENAN. It should be noted in this connexion that DNA is less

Table 5. *Relative specific activities of the DNA extracted from citric nuclei (CN), non-aqueous nuclei (NAN) and NAN extracted with citric acid (ENAN) from the appendix, intestinal mucosa and thymus of a rabbit which had received ^{32}P 2 hr. before killing*

Type of nucleus	Appendix	Mucosa	Thymus
CN	1373	258	434
NAN	1380	402	415
ENAN	1355	385	440

readily extracted from a Schmidt–Thannhauser deoxyribonucleoprotein fraction than it is from a nucleoprotein fraction that has not been subjected to preliminary alkaline incubation, and that DNA is also less readily extracted from NAN than from CN. It may well be that the DNA becomes more firmly bound to the protein during the alkaline incubation stage and that this effect is more pronounced in NAN than in CN.

DISCUSSION

The methods which we have employed for the isolation of NAN are essentially modifications of the procedure of Allfrey *et al.* (1952). Although the preparation of satisfactory specimens is a long and tedious process with rather poor yields, the principle of using a homogenizer in place of the ball mill used by previous authors has appreciably shortened the procedure, provided that reasonably small amounts of material are being handled, and especially when tissues with a high nuclear/cytoplasmic ratio, such as appendix or thymus, are employed. For tissues such as kidney or embryo liver the process is appreciably longer, but the final preparations are satisfactory. On the other hand, we have not been able to obtain satisfactorily clean preparations from adult rabbit liver. Similar difficulties were encountered by Allfrey *et al.* (1952) with rat liver, but they were fairly successfully overcome when the animals were subjected to a fast before killing. Dounce *et al.* (1950), on the other hand, used rat liver for all their experiments on NAN, but the purity of their preparations has been questioned by Allfrey *et al.* (1952).

Our criterion of purity has been morphological examination of stained smears under the microscope, the value of which has been particularly stressed by Allfrey *et al.* (1952), by Stern & Mirsky (1953) and by Kirkham & Thomas (1953). The preparations illustrated in Plate 1 show the ease with which cytoplasmic contamination may be detected by appropriate staining methods and reveal that the final products are free from any obvious cytoplasmic debris.

The main advantage of NAN over nuclei prepared by the usual citric method is that all the nuclear constituents, with the exception of lipids, should be retained. Consequently it is not surprising to find that DNA accounts for a lower percentage of the nuclear dry weight than is found with CN (Table 1). Similar observations have been made for liver nuclei by Dounce *et al.* (1950). Previous authors have not used rabbit tissues, and it is therefore not possible to make a direct comparison of the DNA content of our NAN as shown in Table 1 with the figures of Allfrey *et al.* (1952), who used mainly calf and horse tissues, but when the same tissues are compared in different species our figures are rather lower than theirs.

Dounce *et al.* (1950) found that the RNA content was higher in relation to the DNA content with rat-liver NAN than for CN. Indeed their preparations of NAN contained slightly more RNA than DNA. Allfrey *et al.* (1952), on the other hand, invariably found considerably less RNA than DNA. In our preparations of NAN the estimation of RNA-P was based on the P content of the ribonucleotide fraction obtained by the Schmidt-Thannhauser process, and since this fraction may contain as much as 20–25 % of non-nucleotide P (Davidson & Smellie, 1952), the figures quoted for RNA-P are consequently maximal values. Nevertheless, the separation by ionophoresis of the nucleotides in this fraction has shown that they are the characteristic four nucleotides of RNA and that they are not accompanied by breakdown products of DNA.

In all our preparations of NAN appreciable amounts of RNA were present but, except in those from embryo liver, the RNA-P content was considerably lower than the DNA-P content. In preparations of CN the DNA-P content as a percentage of the dry weight was much greater (Table 1), on account of the lower proportions of other nuclear constituents (e.g. protein), but the RNA-P content was lower than in NAN with the result that the RNA-P/DNA-P ratio was in all cases much lower in CN than in NAN. These figures are compatible with our subsequent observations that citric acid will extract both protein and RNA from NAN.

The values for the protein content of NAN (Table 1) are calculated on the assumption that the

main nuclear constituent other than nucleic acid is protein; they must be regarded only as approximations, since no allowance has been made for the low-molecular-weight constituents, which in any case probably comprise only a small proportion of the total nuclear substance. Our figures are, however, slightly higher than those found by Allfrey *et al.* (1952) in other species.

Since NAN must contain all the acid-soluble constituents of the cell nucleus, it is a matter of considerable importance to examine the substances extracted from them by citric acid. Kirkham & Thomas (1953) found that 0.14M-NaCl extracted 26–44 % of the nuclear substance of NAN from calf thymus and liver, and showed that the major component extracted had the characteristics of a globulin. Dounce *et al.* (1950) found that dilute citric acid at pH 6.0 extracted about 50 % of the dry weight of rat-liver NAN, and Allfrey *et al.* (1952) found that citric acid removed 18–55 % of the protein of NAN. Neither group of workers, however, appear to have examined the citric acid extracts further. In our experiments with NAN from appendix, intestinal mucosa and thymus, citric acid was found to remove a considerable amount (14–23 %) of nuclear protein, which could subsequently be precipitated by TCA. Although the nuclear membrane may not necessarily have the same permeability characteristics in NAN as in a tissue dispersion which has been exposed only to dilute aqueous acid, the structure of the nuclear membrane as revealed by electron microscopy (Watson, 1954) appears to be of sufficient porosity to permit the passage of quite large molecules. Hence it seems highly probable that CN prepared by the conventional method have lost a significant proportion of their original protein content in the process of isolation (see also Dounce, 1955). Such a supposition is supported by the observation that the protein content of individual nuclei in tissue sections as determined by spectrophotometric methods is much higher than that in isolated CN examined in bulk (Pollister & Leuchtenberger, 1949). Consequently, results of studies on proteins obtained from nuclei isolated in dilute aqueous acid solutions must be regarded with some reserve.

Preliminary estimations made by means of interference microscopy by Dr A. J. Hale on nuclei prepared by us from thymus have confirmed a difference in nuclear mass, corresponding to the loss of about 14 % of protein in nuclei treated with citric acid compared with those isolated from non-aqueous solvents.

Citric acid extracts of NAN also contain the non-protein, non-nucleic acid constituents of the nucleus which remain in solution when the extracted protein is precipitated with TCA. Among such constituents are many P compounds, including

nucleotides of adenine, guanine, hypoxanthine, uracil and, in smaller amount, cytosine. An account of our observations on such nucleotides will be given in another paper, but it may be noted here that recent work by several groups of authors (Sacks, Lutwak & Hurley, 1954; Schmitz, Potter, Hurlbert & White, 1954; Schmitz, Hurlbert & Potter, 1954; Smith & Mills, 1954*a, b*) has indicated the general occurrence of mono-, di- and tri-phosphates of adenine, guanine, uracil and cytosine in mammalian tissues. Naora & Takeda (1954) have also found labile phosphate, derived possibly from adenosine triphosphate, in rat-liver NAN. Such compounds, together with RNA, presumably comprise at least part of the ultraviolet-absorbing materials observed, in addition to the expected amount of DNA, in living cell nuclei by Walker & Yates (1952) and more recently by Davies (1954).

The effect of citric acid on the nucleic acids of NAN is also of considerable interest. In our experience citric acid extracts contain no significant amounts either of DNA or of deoxypentose-reacting materials. It is therefore reasonable to conclude that CN may be used with impunity in experiments on DNA. On the other hand, citric acid extracts appreciable amounts of RNA from NAN, and this RNA is precipitated along with protein when TCA is added to CE. About half the RNA present in NAN may, for example, be extracted by citric acid from appendix and thymus nuclei. Since appendix and thymus tissue contain little cytoplasmic RNA in any case and since they yield particularly clean specimens of NAN as judged by microscopical appearance and reproducibility of analytical figures, it seems unlikely that the RNA extracted by citric acid from appendix and thymus NAN is merely cytoplasmic RNA contaminating the preparations. If, however, it is in fact true nuclear RNA, it is obviously distinct in both its solubility properties and its rate of uptake of ^{32}P from the RNA that remains in the ENAN. In nuclei prepared in aqueous solvents, such RNA would in all probability appear on fractionation among the cytoplasmic constituents, and when these constituents are subdivided the RNA would appear in the supernatant (cell sap) material rather than in the particulate fractions. Several authors (e.g. Smellie, McIndoe, Logan, Davidson & Dawson, 1953; Barnum, Huseby & Vermund, 1953) have observed a metabolic distinction between the RNA's of the different cytoplasmic fractions and have suggested that the RNA of the cell sap plays a role distinct from that of the RNA of the cytoplasmic particles. The present results suggest that these observations might be attributed, in part at least, to the presence in the cytoplasm of RNA of nuclear origin.

Since citric acid extracts no DNA from NAN it is not surprising to find that in our experiments with ^{32}P the relative specific activity of the DNA of any one tissue is the same in NAN as in CN in all tissues studied, with the exception of intestinal mucosa. In this tissue the higher activity of the DNA in NAN has been consistently found, and is clearly not due to contamination of the DNA from NAN by a highly active concomitant, since it persists at the level of the separated nucleotides (Table 3). This conclusion is confirmed by the observation that the activity of the DNA is the same in ENAN as in NAN (Table 5). We are unable yet to explain the anomalous position of mucosal DNA, but it must be remembered that in a heterogeneous tissue such as mucosa it is possible that one or other method of isolation of nuclei may preferentially yield the nuclei of one specific cell type. It is also possible that, in the removal of intestinal mucosa from the excised gut, the inevitable time lag before freezing is sufficient to permit a certain amount of autolysis, which continues during the isolation of CN but is stopped at the freeze-drying stage in the preparation of NAN. The action of nucleolytic enzymes might conceivably result in a selective fractionation of part of the DNA, with the loss of diffusible fragments in the aqueous medium. This difference in specific activity may then be an observation of a fractionation of DNA.

The possibility of heterogeneity of the DNA is also raised by the evidence that sodium dodecyl sulphate as employed by Kay, Simmons & Dounce (1952) does not extract all the DNA from the nucleoprotein residue.

The differences in the activities of nuclear RNA from NAN and from CN are large and are not confined to any one tissue. Tyner *et al.* (1953) have observed similar differences in the incorporation of ^{32}P into the nuclear RNA of liver nuclei isolated in citric acid and in ethylene glycol. The nuclear RNA of the former had almost four times the activity of the nuclear RNA of the latter. In the tissues that we have examined the differences are not so great, but when NAN are extracted with citric acid the specific activity of the RNA remaining in the ENAN is greater than that in the NAN although not yet so high as that in the corresponding CN. The specific activity of the RNA extracted by the citric acid is much lower. Since cytoplasmic RNA has a lower relative specific activity than the nuclear RNA from the CN of the same tissue (Smellie *et al.* 1955), the specific-activity measurements could be interpreted as evidence for the contamination of NAN by cytoplasmic material.

Such an interpretation, however, is not in accordance with our observations that the microscopical appearance of stained smears shows no

visible evidence of cytoplasmic contamination and that in some of the tissues which we have used most frequently, e.g. appendix and thymus, the amount of cytoplasm is small, the nuclear cytoplasmic ratio is high and the corresponding NAN are particularly clean in appearance. Indeed sections of these tissues stained with pyronin-methyl green show very little evidence of pyroninophilic material in the cytoplasm.

In the preparation of NAN from tissue homogenates the ratio RNA-P/DNA-P falls as obvious cytoplasmic material is removed, but the limiting value reached in NAN is not lowered by further manipulation. This value is invariably higher than the corresponding value for CN from the same sample of tissue, and in thymus corresponds to the figure found in nuclei isolated from diethylene glycol. It is, moreover, not unreasonable to suppose that if the nuclear RNA is markedly heterogeneous in metabolic activity one fraction of it might have a turnover similar to that of the cytoplasmic RNA's. We are therefore disposed to conclude that the RNA of the cell nucleus is heterogeneous as it exists in nuclei prepared in non-aqueous media and hence in the intact cell. This heterogeneity is manifested both by solubility characteristics and metabolic activity. Although nuclei isolated in dilute acid solutions by conventional procedures can provide useful information, in their preparation part of the nuclear RNA is lost and the study of such nuclei can give only a partial picture of nuclear processes.

SUMMARY

1. A description is given of a modified method for the preparation of rabbit cell nuclei in non-aqueous media. Such non-aqueous nuclei (NAN), which retain their water-soluble constituents, are compared with nuclei (CN) isolated by the conventional citric acid method.

2. On a percentage basis NAN have a much lower content of deoxyribonucleic acid (DNA) than CN have, but their content of ribonucleic acid (RNA) is slightly higher. The protein content of NAN lies between 79 and 92% and is considerably higher than that of CN (66-72%).

3. In experiments on the incorporation of radioactive phosphorus into the cell nuclei of rabbit tissues it was found that the specific activity of DNA was the same in NAN as in CN in all tissues examined except intestinal mucosa where, 2 hr. after administration of ^{32}P , it was invariably higher in NAN than in CN. The activity of nuclear RNA was higher in CN than in NAN in all tissues examined.

4. When NAN were treated with citric acid in conditions similar to those obtaining in the

isolation of CN no DNA was removed, but 14-23% of the protein content was extracted, together with about half the RNA. The RNA so removed had a specific activity considerably lower than that of the RNA which remained.

5. The implications of these observations are discussed, and it is concluded that the RNA of the cell nucleus is heterogeneous in nature, a considerable proportion being lost in a conventional preparation of nuclei from aqueous media. Such nuclei are thus inadequate for a complete study of nuclear metabolism.

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The Metabolism of 3-Hydroxyanthranilic and Quinolinic Acids in the Chick Embryo

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3-Hydroxyanthranilic acid (I) occupies a central position in the biological degradation of tryptophan (for discussion, see Cedrangolo, 1953). It can replace nicotinic acid in the growth of the rat (Wiss, Viollier & Müller, 1949); furthermore, it is readily converted *in vitro* into quinolinic acid (II) (Priest, Bokman & Schweigert, 1951).

Makino, Itoh & Nishi (1951) claimed to have demonstrated *in vitro* the conversion by mammalian liver of 3-hydroxyanthranilic and quinolinic acids into nicotinic acid, but Cedrangolo & Scardi (1951) raised the objection that the long incubation time in these experiments might have induced bacterial growth and therefore yielded unreliable results. In fact, Henderson, Hill, Koski & Weinstein (1951) and Hellmann & Wiss (1952) failed to confirm the results of Makino *et al.* (1951); and Denton, Kellogg, Rowland & Bird (1952) and Cedrangolo, Quagliariello & Della Pietra (1953), after injecting 3-hydroxyanthranilic acid into hen's eggs, were unable to show any formation of nicotinic acid during incubation.

Quinolinic acid also promotes rat growth (Henderson, 1949) and appears in the urine after administration of tryptophan (Schayer & Henderson, 1952), but in experiments *in vitro* Cedrangolo & Jannella (1950) and Cedrangolo & Scardi (1951) were unable to find any conversion of quinolinic into nicotinic acid in homogenates and tissue slices from liver, kidney, spleen, brain and muscle of rats

and rabbits. Additional support for the absence of conversion of quinolinic into nicotinic acid was given by Cedrangolo, Della Pietra & Quagliariello (1953), who found that quinolinic acid, injected into hen's eggs, did not cause any increase in the content of nicotinic acid during incubation.

Given this conflicting evidence, it was thought worth while to examine whether 3-hydroxyanthranilic acid, and possibly quinolinic acid as well, could be converted into nicotinic acid by chick embryo. Short preliminary communications of the results referred to in this paper have already appeared elsewhere (Quagliariello & Della Pietra, 1954; Della Pietra, Quagliariello & Auricchio, 1954).

EXPERIMENTAL AND RESULTS

We first investigated whether 3-hydroxyanthranilic acid could be converted into nicotinic acid.

Chick embryos on the 12th incubation day were homogenized in a Potter glass homogenizer with Krebs-Ringer-phosphate solution (5 ml./g. tissue). The incubation mixture consisted of 5 ml. of homogenate, 3 mg. of 3-hydroxyanthranilic acid (Hoffman-La Roche and Co.), and Krebs-Ringer-phosphate, pH 7.3; total vol. 15 ml. The incubation took place at 37° for 3 hr., with oxygen as the gas phase. In order to autolyse the tissues, the incubation was then continued under benzene for 21 hr. The volume was then brought to 40 ml. with water, and, after autoclaving for 20 min. at 121°, the volume was readjusted to 40 ml. and the suspension filtered. Samples of the filtrate were taken for the quantitative analysis of nicotinic acid by the microbiological technique of Della Pietra & Quagliariello (1953), using *Lactobacillus arabinosus*. Blanks with tissue only (A), with 3-hydroxyanthranilic acid only (B), and with tissue + 3-hydroxyanthranilic acid added at the end of incubation (C), were run in parallel through the entire procedure. Values of A and C were always identical; values of B were nil.