Technical Papers

Effect of Nuclear Counting Procedures on Determination of the Desoxypentosenucleic Acid Content of Rat Liver Cell Nuclei[1]

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Values of $5.9–14.0 \times 10^{-6}$ μg DNA/liver cell nucleus have been reported (*1–8*) in young mature rats (200–300 g). Such wide variation might conceivably result from differences in the media used in isolating nuclei, in nuclear counting techniques, or in the method of determining DNA. The work of Schneider (*9*) and of Davidson (*10*) would appear to rule out the last possibility.

While attempting to devise a medium from which clean nuclei and cytoplasmic particles could be obtained simultaneously, observations were made that were originally interpreted to indicate that the composition of the medium determined the amount of DNA finally calculated to be present in nuclei. When rat liver nuclei were prepared in a modified citric acid medium, a value of $10.4 \pm 0.59 \times 10^{-6}$ μg DNA/nucleus (9 determinations) was obtained. When, however, nuclei were isolated from a sucrose medium, the value was $7.7 \pm 1.2 \times 10^{-6}$ (3 determinations).

During the course of these experiments, it was observed that considerable numbers of nuclei from the final preparations made in the modified citric acid medium adhered to the walls of the glass pipettes used for transferring samples to the counting chambers. This did not occur when nuclear counts were made on initial homogenates (prior to the isolation of the purified nuclei) prepared either in citric acid or in sucrose. The adherent nuclei were firmly attached to the glass and were removed with considerable difficulty in the cleaning process. Special care in cleaning the glass pipettes prior to use did not reduce the tendency to adhere. The importance of this as a source of error becomes apparent when it is realized that adherence of large numbers of nuclei to the glass pipettes would result in abnormally low values for the numbers of nuclei present in the suspensions and in an erroneously high estimation of DNA/nucleus. This may account for some of the variations reported in the literature. The use of concentrated acid, solvents, fixatives, or silicone coating of the pipettes did not prevent the adherence of nuclei.

The possibility of evaluating this "sticking" of nuclei as a source of error by substituting a wire loop[2] for the pipette was suggested by Caroline Raut, microbiologist, of this institute. Male rats of the Fisher strain, weighing 210–230 g, were anesthetized with ether, the livers perfused *in situ* with ice-cold isotonic saline, and pulped by forcing them through a plastic tissue press. They were then homogenized in 1–2 volumes of the medium (to be described), using a motor-driven metal pestle covered with rubber and fitted to a glass tube, and the homogenate diluted to 10 volumes with the medium (subsequently referred to as the initial homogenate). A 2 ml sample of this homogenate was removed with a glass pipette and diluted to 5 ml with the medium. A drop was then placed on a hemocytometer and allowed to spread under the coverslip. For each sample a minimum of 1000 nuclei was counted in 10 hemocytometer fields of 9 RBC squares each, using a phase contrast microscope. The standard error of all homogenate and final nuclear preparation (loop and glass pipette) counts made in these experiments was $2.8 \pm 0.24\%$. DNA was determined as previously described (*11*).

Nuclei were isolated, using a medium of either 0.026 *M* citric acid or 0.25 *M* sucrose. Centrifugations were carried out in an International refrigerated centrifuge, Model PR-1, and all operations were performed in a cold room at 0° C. When citric acid was used, the initial homogenate, after a portion had been removed for nuclear counts, was centrifuged for 5 min at $690 \times g$. The supernatant was discarded, and the residue was resuspended in 0.026 *M* citric acid and recentrifuged for 3 min at $16 \times g$.[3] The residue (R2) was saved, and the supernatant was recentrifuged under the same conditions. The supernatant (S3) was saved, and the residue pooled with residue R2, resuspended and centrifuged for 3 min at $28 \times g$. The residue was discarded, and the supernatant recentrifuged for 3 min at $16 \times g$. This residue was discarded, the supernatant was pooled with supernatant S2, and centrifuged for 5 min at $690 \times g$. The resulting supernatant was discarded, the residue resuspended and recentrifuged for 5 min at $690 \times g$, and the supernatant discarded. A yellow layer on top of the residue, consisting largely of cell debris, was swirled off gently; the remaining residue was considered the final nuclear preparation. It consisted of nuclei, a small amount of nuclear membranes, and occasional bits of nuclear and nonnuclear fragments. This preparation was diluted to 20 ml, and a 1 ml aliquot was used for counting; the suspension was transferred to the counting chamber with a glass pipette. When the wire loop was used in the counting, a loopful of the nuclear preparation was introduced under the coverslip of the hemocytometer and 5 RBC squares were counted. In 8 such fields a minimum of 5000 nuclei was enumerated. The DNA

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[2] The loop was made from a short length of wire and consisted of several decreasing spirals at one end and a short handle at the other.

[3] To obtain low speeds an accessory rheostat was placed in series with the standard control of the refrigerated centrifuge (*12*).

TABLE 1

DESOXYPENTOSENUCLEIC ACID CONTENT OF RAT LIVER CELL NUCLEI*
(Medium: 0.25 *M* Sucrose)

Diluting medium for counting	DNA values observed 10^{-6} μg/nucleus)
0.25 *M* sucrose	7.9
	8.8
	8.0
0.25 *M* sucrose plus 0.026 *M* citric acid	7.6
	8.2
	7.9
Grand Av	8.1 ± 0.17 SE

* Nuclear counts were made on the initial homogenates.

content of the remaining material was then determined.

When sucrose was used, the initial homogenate, after a portion had been removed for nuclear counts, was centrifuged for 10 min at $690 \times g$. The supernatant was discarded, and the residue resuspended and recentrifuged under similar conditions. The residue obtained was considered to be the nuclear preparation, and its DNA content was determined. Inasmuch as it is very difficult to see and count the nuclei in the initial homogenates when diluted with 0.25 *M* sucrose, a mixture of 4 ml of 0.25 *M* sucrose and 1 ml of 0.026 *M* citric acid was employed. The latter made the nuclei more prominent and more readily counted, but had no effect on the DNA/nucleus (Table 1).

counting method (Table 2) was appreciably lower than the 70–90% recovered by Hogeboom *et al.* (*13*) using a sucrose medium and a filtration technique. In the present experiments, a higher nuclear yield could have been obtained in the citric acid technique if the yellow layer had been allowed to remain with the nuclei, but a less satisfactory preparation would have resulted.

Attempts were then made to determine whether clean and nonclumped nuclei isolated from sucrose also adhere to the walls of the glass pipettes. Experience has shown that nuclei prepared in sucrose have a tendency to clump and are therefore very difficult to count. Several procedures have been suggested to eliminate this clumping and its attendant nonnuclear contamination. The most promising are the layering technique of Wilbur and Anderson (*12*) and the $CaCl_2$-sucrose-filtration technique of Schneider and Petermann (*14*). However, the nuclear preparations obtained by Wilbur and Anderson, although clean, contain clumped nuclei, and the procedure of Schneider and Petermann subjects the nuclei to the stress of vacuum and passage through closely woven cloth. A procedure was used which combined features of each method. The medium consisted of 0.25 *M* sucrose and 0.0009 *M* $CaCl_2$, and the nuclei were isolated by layering. Nuclear counts were made on the initial homogenates using glass pipettes, and on the final nuclear preparation using both pipettes and wire loops. On microscopic examination of the pipettes used in transferring the final

TABLE 2

DESOXYPENTOSENUCLEIC ACID CONTENT OF RAT LIVER CELL NUCLEI*
(Medium: 0.026 *M* Citric Acid)

Medium	No. of observations	Nuclei recovered (%)		DNA values observed (10^{-6} μg/nucleus)	
		Loop	Pipette	Loop	Pipette
Citric acid†	5	63 ± 4.2	41 ± 2.3	7.5 ± 0.2	11.2 ± 0.1
Sucrose-$CaCl_2$	1	74	54	7.1	9.2

*Nuclear counts were made on both initial homogenates and final nuclear preparations.
† Figures represent average values and standard errors.

When the wire loop was used in transferring nuclear material to the counting chamber, the DNA values/nucleus of preparations made from citric acid were quite close to those obtained with nuclei prepared from sucrose (Tables 1 and 2). Whether the small difference observed ($P = 0.05$) was due to differences in media or to other factors is not known. On the other hand, when glass pipettes were used, considerably more DNA was estimated to be present than in both the citric acid–prepared nuclei counted using a wire loop ($P < 0.001$), and those obtained from sucrose ($P < 0.001$) (Tables 1 and 2). Furthermore, the recoveries of nuclei in the final citric acid preparation were lower than those obtained when using the wire loop ($P < 0.01$) (Table 2).

The recovery of 63% of the citric acid–prepared nuclei in the final nuclear preparation with the loop-preparations, nuclei were again found to adhere to the walls. The estimated DNA/nucleus and the nuclei recovered are indicated in Table 2.

These experiments demonstrate that the use of glass pipettes as part of the procedure for counting rat liver cell nuclei in final nuclear preparations employing a medium of either citric acid or sucrose is contraindicated. The use of a wire loop circumvents the counting difficulties encountered with pipettes. When pipettes were used with the citric acid medium, the observed value of DNA/nucleus was $11.2 \pm 0.1 \times 10^{-6}$ μg. On the other hand, when a wire loop was used with the citric acid medium, the observed value of DNA/nucleus was $7.5 \pm 0.2 \times 10^{-6}$ μg. The latter value is quite close to the value of $8.1 \pm 0.17 \times 10^{-6}$ μg DNA/nucleus observed when sucrose was used as the medium, and the counts were done on the initial homogenates.

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Biosynthesis of C^{14}-Specifically Labeled Cotton Cellulose[1]

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Cellulose is formed biologically by a process of which the chemistry is almost unknown. In 1949 the author conceived the idea of synthesizing position-labeled bacterial cellulose from sugars labeled specifically with radioactive C^{14}. It was considered that a study of radioactive labeled celluloses synthesized by the microorganism *Acetobacter xylinum*, cultured on, or supplied with, C^{14}-specifically labeled substrates, might help to elucidate the mechanism of normal cellulose biosynthesis. Accordingly, arrangements were made for collaboration with H. S. Isbell and his associates at the National Bureau of Standards and with Milton Harris and his associates at the Harris Research Laboratories. A program was formulated for a joint project, and the Atomic Energy Commission awarded the funds for the proposed basic research. Four reports or publications have resulted from the synthesis of radioactive-labeled sugars and of C^{14}-labeled bacterial cellulose, two from the former (*1, 2*) and two from the latter (*3, 4*).

With the study of specifically labeled bacterial cellulose well under way, the author planned some experiments to learn if radioactive cellulose could be produced in the *Gossypium herbaceum* cotton boll. It is the purpose of this paper to report the success of the experiments concerned with the biosynthesis of C^{14}-specifically labeled cellulose in a cotton boll, while attached to the plant.

Approach. Previous determinations by the author, as well as by other workers, had indicated that the reducing-sugar content of the cotton boll is 60–70% between the time of pollination of the flower and 21 days following fertilization. Likewise, the literature (*5*) showed that the primary cell wall of the cotton fiber is formed and reaches its full length during the first 20 days or so following fertilization of the flower. With this information available, it was reasoned that, if D-glucose-1-C^{14} were introduced into the cotton boll through the vascular system of the plant at the time of optimum sugar translocation and cellulose synthesis, it would diffuse with the other sugars and enter into the cellulose formed by the boll. The importance of introducing the labeled sugar just at the time of optimum sugar translocation cannot be overemphasized. The best results from the standpoint of glucose-1-C^{14} utilization should be obtained at the time the greatest quantity of reducing sugars is being converted to cellulose in the normal boll. This, according to the best prevailing knowledge of cellulose formation, should be approximately 21 days after fertilization of the flower.

Procedure and Results. Four Stoneville 2 B cotton plants were grown from seed in 6-in. pots containing soil in a greenhouse of the U. S. Department of Agriculture at Beltsville, Md. The plants grew and flowered normally. Considerable experimentation was required to develop the best procedure for introducing glucose into the boll without hindering its development. The procedure selected was to slice the stem longitudinally just below the boll with a razor blade and place the cut portion into the sugar solution. The soil in which the plant was growing was first allowed to dry to the point that the plant lost some turgor and was then watered approximately 30 min before introduction of the glucose-1-C^{14}. The sugar solution is taken into the boll within a few minutes, the rate of transfer varying with the sugar content of the boll at the time of experiment. The stem and the boll were carefully taped to prevent injury and to allow normal boll development.

One experiment was performed by introducing the glucose-1-C^{14} into the boll (ovary) the same day the flower was pollinated and harvesting the developing boll on the 21st day. The cotton fibers were removed, dewaxed (*6, 7*), and extracted with 1% NaOH. The small quantity of primary-wall cellulose remaining was assayed for radioactivity. The radioactivity of the cellulose itself was low; the extracts taken of the cellulose and of the remaining parts of the cotton boll contained most of the radioactivity introduced as glucose-1-C^{14}.

Accordingly, a second experiment was performed in which 12.5 μc of glucose-1-C^{14} were introduced

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