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The Nucleoprotein Content of Fibroblasts Growing *in vitro*

4. CHANGES IN THE RIBONUCLEIC ACID PHOSPHORUS (RNAP) AND DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNAP) CONTENT

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In the earlier papers of this series (Davidson & Waymouth, 1943, 1945, 1946) an account has been given of the conditions under which it is possible to record an increase in the nucleic acid content of chick-heart fibroblasts growing *in vitro*. Using the roller-tube technique of Willmer (1942), appreciable increases in the total nucleic acid phosphorus (NPP) of the fibroblasts have been obtained when the cultures were planted in fowl plasma and allowed to grow in chick-embryo extract. Defatted chick-embryo extract and fowl plasma, either together or separately, produced smaller increases in nucleic acids measured over 48 hr. periods, and led to a more rapid deterioration of the cultures (Davidson & Waymouth, 1946). Loss of nucleic acid was shown to occur over the 2 days following planting, and was greater for cultures in Tyrode solution than for those in embryo extract. However, the increase following addition of embryo extract to the cultures in Tyrode was as great 2 days later as in the case of cultures provided with embryo extract throughout. As a rule increases in NPP were greater for higher than for lower concentrations of embryo extract.

Schmidt & Thannhauser in 1945 published their method for determining the amounts of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in tissues (Schmidt & Thannhauser, 1945). This method has been adapted for estimating the phosphorus content of each acid after separation, and it has been possible to follow changes in these two components in the fibroblast cultures, and to investigate the relationship between growth and the total nucleic acid phosphorus (NPP), the ribonucleic acid phosphorus (RNAP) and the deoxyribonucleic acid

phosphorus (DNAP). Although Willmer (1942) and Cunningham & Kirk (1942) proposed that growth in tissue cultures could be measured by determining the NPP content of cultures at different stages of development, they did so before it had become clear that there were two types of nucleic acids in each cell, the more abundant one usually being the ribonucleic acid of the cytoplasm and nucleolus, the other, the deoxyribonucleic acid, being apparently always confined to the nucleus.

In the tissue-culture technique which we have employed, it is possible to select a basal starting level of NPP according to the number and size of the cultures employed. From the time when growth-promoting medium is added to the cultures, changes in the amounts of phosphorus from both types of nucleic acids can be measured at varying intervals of time. Since the tissues are in a resting state before addition of growth-promoting medium, and the production of new tissue is in abeyance, it is reasonable to suppose that if one nucleic acid is the precursor of the other, then it will be the first to appear in the growing cultures. In this work we have been able to show that an increase in the RNAP always precedes by relatively long intervals any increase in DNAP. If the assumption that the P content of the nucleic acids is invariable is justified (as has been questioned; Wiame, 1946), the synthesis of RNA appears to be established before that of DNA.

Our observations on the changes occurring in both the RNAP and the DNAP during growth of fresh embryo-heart cultures have confirmed the general findings of Brues, Rathbun & Cohn (1944) and have enabled us to find satisfactory conditions for growth

with the roller-tube method. Although with our original technique an increase in RNAP could always be obtained in growth-promoting media, a sustained and substantial rise in DNAP only occurred when the modification was introduced of cutting the individual explants small enough to reduce to a minimum loss of material by cell destruction at the centre.

METHODS

Fresh explants of 12-day chick-embryo heart were grown in plasma clot in roller tubes (Willmer, 1942), the technique which has been employed in the earlier experiments of this series (Davidson & Waymouth, 1943, 1945, 1946). Each roller tube contained 24 pieces of tissue in three rows of eight and embedded in clots of 0·1 or 0·05 ml. plasma per row according to the size of cultures employed. The growth-promoting medium was either embryo extract alone or a mixture of embryo extract and serum. In each case 0·5 ml. was allocated to each roller tube, and in experiments running over long periods, the fluid phase was renewed every 24 hr. Preliminary experiments showed that in control cultures in Tyrode solution the RNAP and DNAP fell to a steady level, which we have termed the resting state. In all the subsequent tests, the cultures were maintained for at least 20 hr. in Tyrode solution before the addition of growth-promoting medium.

Preparation of growth-promoting media. Embryo extract (EE) was prepared from 12-day chick embryos by the method already described (Davidson & Waymouth, 1943) (total N, 50–90 mg./100 ml.). In the preparation of the embryo extract-serum mixture (EES), 4 ml. of the original concentrated extract and 2 ml. of cockerel serum were diluted to 10 ml. with Tyrode solution (final N concentration *c.* 60 mg./100 ml.). Owing to the variations in plasma composition and in the concentration of embryo extracts prepared in the course of different experiments, comparisons of results are only made under the fairly uniform conditions of one particular experiment.

Estimation of RNAP and DNAP in the tissues. The two nucleic acid fractions were separated by the method of Schmidt & Thannhauser (1945), and the P content of each determined by the method of Berenblum & Chain (1938) as modified by Davidson & Waymouth (1943). It was possible to detect changes in the amount of P of the order of 0·1 μg. over the range of 0·1–8·0 μg. P, and determinations were carried out on the contents of one or two roller tubes according to the amount of tissue and plasma employed in the particular experiment.

The methods of extraction and separation of the nucleic acids were the same in every case. The fluid phase was discarded and the tube washed out with a small volume of 0·9 % saline. Two ml. of ice-cold 10 % (w/v) trichloroacetic acid (TCA) were added to each tube, and the tissues and plasma scraped off the sides of the tube. The contents were transferred to 15 ml. graduated pyrex tubes, and centrifuged rapidly. The roller tube was washed out and the tissue extracted twice more with 2 ml. portions of TCA, the whole operation being carried out within 20 min. to avoid any loss of nucleic acid by hydrolysis; in this way, only acid-soluble P was removed by the TCA treatment. Extraction of the lipid P was completed in a series of extractions using 80 % and

absolute ethanol, two treatments with chloroform-ethanol (1 : 3) mixture at 70–80°, and finally ether. In most cases the extracts were discarded after centrifuging, and only in a few experiments were acid-soluble and lipid P determined. The P in the dry powder resides mainly in the nucleic acids, since the amount of phosphoprotein in this tissue has been shown to be negligible.

For the separation of RNAP and DNAP, the residue was incubated at 37° overnight in the centrifuge tube with 1 ml. of silica-free N-NaOH, and the unhydrolyzed DNA separated from the hydrolyzed RNA by addition of 1·5 ml. distilled water, 0·5 ml. 2·5 N-HCl, and 0·6 ml. of 30 % (w/v) TCA. The DNA-containing precipitate was spun down by centrifuging rapidly for 15–20 min.; the supernatant liquid (the RNA fraction) was transferred to a short, wide-necked, pyrex boiling tube. The precipitate was washed twice with 0·5 ml. portions of 5 % TCA, which were added to the supernatant fluid. After adding 0·3 ml. 60 % (w/w) HClO$_4$, the solution was evaporated at 100°, before transferring to an air bath in which complete oxidation of the tissue was carried out at a temperature of about 220°. The precipitate containing the DNA was oxidized in the same way with 0·2 ml. of HClO$_4$. P was now estimated in both sets of materials, the results being corrected for reagent blanks, which were determined for each experiment.

Method of expressing results. In each experiment there were usually 14 roller tubes each containing 24 pieces of tissue in 0·3 or 0·15 ml. of plasma, and 6 'plasma blanks' containing only the appropriate amounts of plasma, clotted by the addition of small pieces of heart tissue, which were subsequently removed. In most experiments, a pair of tissue tubes and a plasma blank were used for RNAP and DNAP determinations at zero time and at selected intervals during the growth period. When it was found that over certain periods there was little change in the plasma blanks, it was possible to dispense with intermediate samples and to correct some of the tissue estimations with mean or intermediate values.

It has not been possible to evolve a statistical basis for significance in the course of this work owing to variations in design and conditions from test to test. Moreover, there is an unknown error involved in the use of the plasma blank in the correction of the tissue figures, since an appreciable portion of the plasma clot is used up by the growing tissue as it extends its area, while there is no comparable alteration in the plasma tubes. Our practice of correcting for plasma blanks is likely to reduce the tissue values more than necessary, and so lead to an underestimation of the real increase in tissue P. It seems reasonable under these circumstances to consider these final increases in tissue P significant only when they equal or exceed the corresponding increases in the plasma blanks.

The initial values of both RNAP and DNAP in the tissue tubes and in the plasma tubes depend on the number and size of the cultures, and the composition of the plasma. Variations in the plasma blanks arise from the use of different batches of fowl plasma throughout these tests. The RNAP and DNAP contents per tube are produced by 24 relatively large pieces of tissue (12–15 mg. fresh weight), while the contents per 2 tubes come from 48 much smaller pieces of tissue.

An examination of the RNAP figures (Table 1) shows that the real tissue increments (i.e. the values obtained after correction has been made for the corresponding plasma

blanks) are in every case considerably greater than the amounts of RNAP taken up by the plasma clots from the embryo extract or embryo extract-serum mixture. Both the tissue and plasma RNAP values, of course, increase as the duration of the growth period is extended, the amount of this increment depending on the concentration of the EE or EES. As these concentrations of extract are kept fairly uniform throughout each test, the change in the plasma blanks taken at different times is gradual, and their RNAP values do not fluctuate appreciably. The error involved in the actual determination of P by the method employed was of the order of 0·1 μg. P, and it is considered that all the tissue RNAP increments shown in Table 1 are significant.

In the first group of tests in which the DNAP per tube of 24 cultures was measured, a significant increase is only found in tests 40, 49 and 50 (Table 2). In the others of this group there is very little change in the DNAP content, or there is a definite decrease which may be related to the relatively high content of the corresponding plasma blank, for which correction has already been made. Significant increases in DNAP are, however, obtained in the second group in which smaller explants (tests 56, 58, 59 and 62) are used, and while the plasma blank apparently falls in test 56, it increases considerably in tests 58, 59 and 62. The initial plasma value is unusually high in test 56.

RESULTS

The earlier work (Davidson & Waymouth, 1943, 1945, 1946) has shown that fresh chick-heart cultures lose an appreciable amount of their nucleic acid P (NPP) when they are maintained in Tyrode solution for 2 or 3 days after planting. This fall in total NPP was steep over the first 24 hr. and more gradual over later periods. It also occurred when the cultures were maintained in embryo extract from the start, but in this case the drop in NPP content was not so large.

In the present work the changes which occur in the RNAP and DNAP contents of the cultures under the conditions described above have been investigated. Immediately after planting, the ratio RNAP/DNAP was found to lie between 2·2 and 2·8. If the cultures were maintained in Tyrode solution for 1 or 2 days, there was a loss of both RNAP and DNAP in amounts which left the ratio RNAP/DNAP much the same as in the freshly planted tissue; for example, for 12 tests the average figure for the ratio at this time was 2·25. This suggests, if anything, a slightly greater reduction in the RNAP than in the DNAP.

By the end of 20 hr. in Tyrode solution, the removal of this easily lost nucleic acid was almost complete, and the tissues could be said to have reached their resting state (Fig. 1). Since the loss involved both nucleic acids in the ratio of their occurrence in fresh tissue, it could be assumed to arise from the breakdown and washing out of cells damaged in the process of cutting the heart tissue. The simultaneous fall in the RNAP and DNAP also occurred if embryo extract was used instead of

Tyrode solution in the period immediately following planting. That it was not so great as the fall occurring in Tyrode solution can be seen in Fig. 1. If, after the first 20 hr., the Tyrode solution or extract was replaced by fresh extract, a visible increase in area of the cultures occurred, and it was accompanied by an increase in RNAP but not in DNAP. The final figure for RNAP is similar for both sets, although at the start of the second 21 hr. test period the cultures which had been in embryo extract from the beginning had an appreciably higher RNAP content.
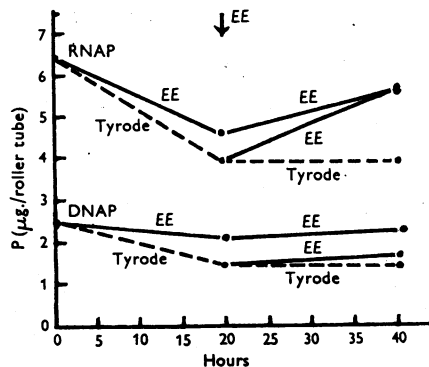

Fig. 1. Changes in ribonucleic acid P (RNAP) and deoxyribonucleic acid P (DNAP) of chick-heart explants *in vitro*, using either Tyrode solution or embryo extract (EE) as the fluid phase. The figures for P represent the amounts found in the two nucleic acids in 24 pieces of tissue (planted in three rows of eight) per roller tube. The continuous line shows the changes which occur when the fluid phase was embryo extract, and the arrow indicates the time of the addition or renewal of EE. The broken lines show the changes in RNAP and DNAP occurring in Tyrode solution. The results at zero time refer to the cultures at the time of planting; at 20 hr. those in Tyrode solution have reached their resting levels of RNAP and DNAP.

As a result of the experience gained in a few experiments of this nature, our practice in later experiments was to maintain the cultures for 2 days in Tyrode solution when changes in nucleic acid content were to be determined over relatively short periods. In experiments of long duration, lasting up to 7 days, it was considered sufficient to keep cultures for 20–24 hr. in Tyrode solution before adding the growth-promoting medium.
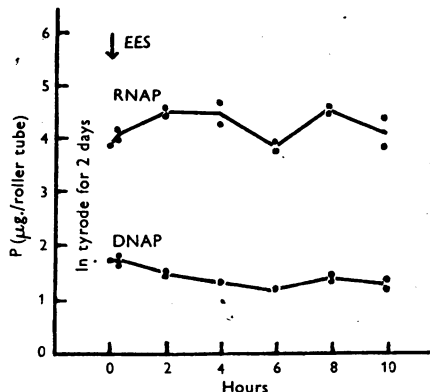
Changes in RNAP and DNAP content at short intervals over the first 48 hr. in growth-promoting medium

The results of some of the preliminary experiments suggested that larger increases in NPP were obtained when the growth-promoting medium was a mixture of embryo extract (12-day chick embryo)

and fowl serum. This medium (EES) was employed throughout the series of tests to be described. The size of the individual cultures was relatively large,

Figs. 2–8. The results are based on the contents of roller tubes, each containing 24 pieces of tissue, and have been corrected for plasma blanks. Most points are the mean of duplicate determinations. In some figures, RNAP/DNAP ratios are given at certain stages.

Fig. 2. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over the first 10 hr. following the addition of a mixture of embryo extract and cockerel serum (EES). At zero hour, the cultures are in their resting state, and the arrow shows when the Tyrode solution was replaced by EES.
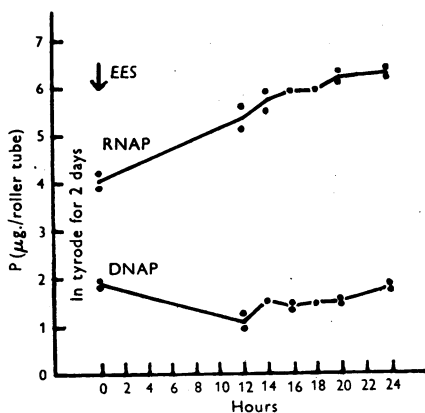

Fig. 3. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over the first 24 hr., following the addition of EES. After planting, the cultures were maintained in Tyrode solution for 2 days, and the arrow shows when this was replaced by EES. The changes were followed at intervals of 2 hr. in the second 12 hr. period.

as compared with the pieces used in the final series of tests. An estimate of the weight of tissue in each roller tube at the start, based on the assumption that the DNAP content is the same as in the original chick heart (15 μg. P/100 mg.) (Davidson & Leslie, 1948), gives an average figure of 12–15 mg./24 tissue pieces.

In the first 12 hr. in EES there was little or no visible change in the size of the cultures, and only a slight rise if any in RNAP (Fig. 2). The DNAP remained unchanged, or fell during the same period.
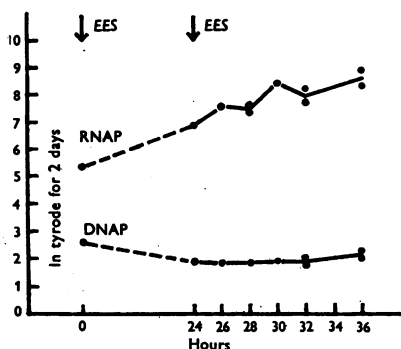

Fig. 4. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over the third 12 hr. period following the addition of EES. The cultures were allowed to come to their resting state in Tyrode solution in 2 days, and the arrows show when the EES was added or renewed. The broken lines indicate that the time scale in the earlier stage has been condensed.
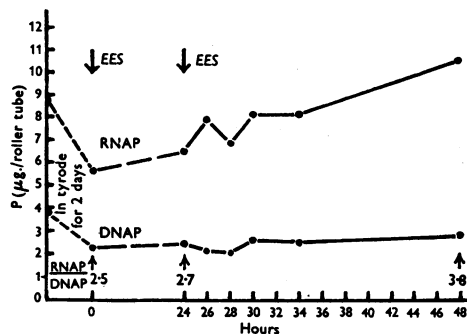

Fig. 5. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over a period of 48 hr. following addition of EES. These changes were followed at intervals of 2 hr. in the third 12 hr. The fall in RNAP and DNAP before zero hour occurs when the cultures are in Tyrode solution. The arrows show when EES was added or renewed.

The amounts of both nucleic acids, determined at intervals of 2 hr., showed regular fluctuations, which were unexplained. They were not, however, thought to be random experimental variations in view of the fact that this and similar curves are based on duplicate determinations, whose values in most cases lay close together. In the case of RNAP values, fluctuations of 0·5 μg. were considered significant if the duplicates agreed, since the corresponding

changes in the plasma blanks were only of the order of 0·1 μg. P. There was very good agreement between DNAP duplicates, and, in these, changes of 0·25 μg. P can be considered significant.
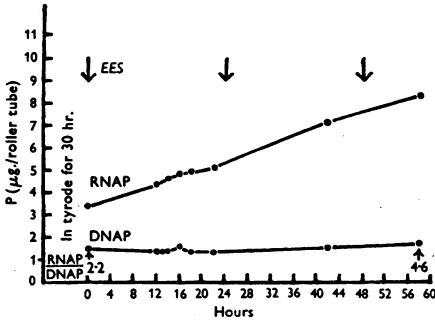

Fig. 6. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over 58 hr. following the addition of EES. These changes were determined at intervals of 2 hr. over the second 12 hr. After planting, the cultures were maintained in Tyrode solution for 30 hr., and the arrows show the times of addition or renewal of EES.
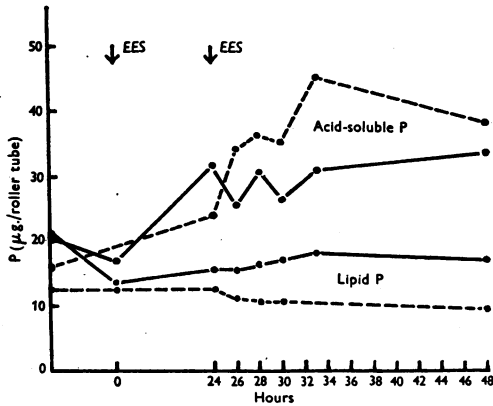

Fig. 7. Changes in acid-soluble P and lipid P in relatively large chick-heart explants growing *in vitro* over 48 hr. following the addition of EES. The conditions of the test are the same as in Fig. 5, but the results for both the tissue tubes (unbroken lines) and the plasma blanks (broken lines) are given on separate curves, as the acid-soluble P is very much less in the tissue tubes than in the plasma tubes in the later stages. The arrows show the times of addition or renewal of EES.

A visible increase in the area of the cultures was observed in the first 24 hr. in contact with the growth-promoting medium (as shown in Pl. 1). In Fig. 3 the RNAP increased appreciably over the same period, while the final DNAP content was much the same as at the start. The fall in the latter over the first 12 hr. was confirmed; in this particular test it was accompanied by a large increase in the RNAP. In the second 12 hr. period the RNAP seemed to increase evenly without any evidence of

the fluctuations which were characteristic of its behaviour in the earlier period. At the same time there was a definite increase in DNAP, but it is doubtful if the rise which occurred between 12 and 14 hr. was of significance.

The marked rise in RNAP unaccompanied by a corresponding rise in DNAP calls for special comment. It was, of course, reflected in a rise in the RNAP/DNAP ratio from 2·2 to 4·2 over the 24 hr., a feature common to all the tests in which relatively large pieces of tissue were employed. This failure of
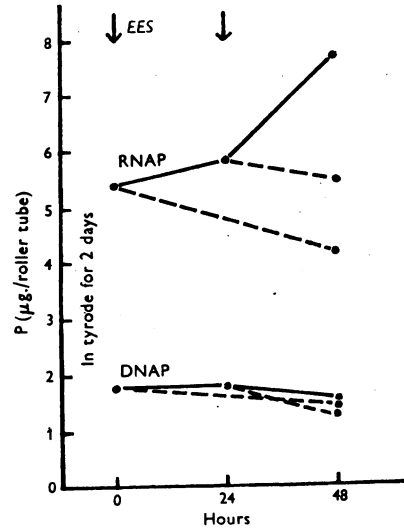

Fig. 8. Effect of colchicine (1 in 4,000,000) on the changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro*, using EES as the fluid phase. The broken lines show the changes occurring when colchicine is present in the EES. After maintaining the cultures in Tyrode solution for 36 hr., EES or EES containing colchicine is added to the tubes at the times indicated by the arrows.

the DNAP to show any significant increase was perplexing at the time, in view of increases in RNAP and in the area of the cultures.

In Fig. 4 are shown the results of a test on the changes occurring in the third 12 hr. period. There was an unusually large fall in DNAP in the first 24 hr. Visible increase in area, continued, however, in the next 12 hr. and the RNAP increased, although there was little change in DNAP. Again, regular fluctuations in RNAP were in evidence following the addition of growth-promoting medium. As similar patterns of RNAP changes were obtained in other tests they cannot be dismissed as random variations (see Fig. 5). On the other hand, it is difficult to understand their significance, and it can only be recorded that the RNAP did not increase evenly over certain periods of growth.

In Fig. 5 the initial drop in both fractions which occurred when the cultures were maintained in Tyrode solution for 2 days is again shown. In this test the changes over the first 24 hr. were not irregular; the RNAP increased significantly, and there was a very slight rise in DNAP. The fluctuations following the second addition of EES showed the same features as before, with the rise in DNAP occurring in the later part of the first 12 hr. period. Although small, these DNAP rises are considered significant, since the corresponding plasma-blank increases were very low or negligible in comparison with the increases in the DNAP of the tissues. At the end of 48 hr. growth, the RNAP had increased 87 % over its initial resting value, while the DNAP had increased by only 18 %. By this time the cultures were very extensive (Pl. 1 C), and the rise in DNAP was, therefore, surprisingly small. The ratio RNAP/DNAP rose over the same time from 2·5 at the resting level to 3·8 after growth.

This increasing ratio, associated with a failure of the DNAP to show an appreciable increase, is a feature of another test, in which measurements were made over a 58 hr. period (Fig. 6). In this case, the RNAP increased by 166 % as compared with a 16 % increase in DNAP, or, expressed in terms of the ratio RNAP/DNAP, there was a rise from 2·2 to 4·6. Fig. 7 shows the results of the determination of acid-soluble and lipid P which were made in the course of a test described above (Fig. 5). As the acid-soluble P in the plasma blanks was higher than in the tissue tubes it was not possible to plot the corrected figures as in the case of RNAP and DNAP contents. Instead the values for tissue tubes and plasma blanks were both drawn on the same graph. The amounts of acid-soluble and lipid P increased in the tissues during growth of the cultures, but only the latter could be considered to increase in the tissue itself. Apparently, the acid-soluble P of the extract and plasma fell considerably in the tissue tubes during the same period.

Inhibition of growth of fibroblasts by colchicine

Colchicine in concentrations of 1 in 20–30 million has been shown to arrest mitosis in growing tissues in vitro (cf. Bucher, 1940). This provided us with a means of confirming that increases in RNAP and DNAP in the presence of growth-promoting media were due to synthetic processes associated with the growth of new tissue.

The results of a typical experiment with colchicine are seen in Fig. 8. The EES was divided into two 8 ml. portions. To one was added 1 ml. of a sterile Tyrode solution containing colchicine (1 in 400,000). The volume was made up to 10 ml. with Tyrode solution. The 8 ml. of EES for the control experiments were similarly diluted with Tyrode.

At the start of this test the majority of the roller tubes received the normal EES, and the remainder contained EES with colchicine (1 in 4,000,000). In the latter both the RNAP and DNAP values showed a distinct fall from the resting values at the end of 48 hr. There was, moreover, no visible sign of growth. In the others maintained in normal EES, good growth was observed, and the RNAP increased appreciably. When normal EES was replaced at the end of 24 hr. by EES containing colchicine the RNAP and DNAP again fell during the next 24 hr. and no further extension of area was observed.

The lower RNAP and DNAP of the cultures maintained in extract plus colchicine confirms that synthesis of these compounds is taking place in the presence of normal EES. In the case of the DNAP this is true even when no actual increase is observed in the growing cultures.

Changes in RNAP and DNAP over long periods of growth

In view of our failure over relatively short periods to obtain a rise in DNAP, commensurate with the rise in RNAP or the increase in area of the cultures, changes in RNAP and DNAP were followed over a period of 6–7 days. As in the earlier tests there was some indication that the rise in RNAP always precedes a rise in DNAP, it was thought at the outset that increases in DNAP might be greater at a later stage of development.

In the course of these tests of longer duration the EES had to be changed six or seven times, and care was taken to eliminate any sediment from the extract by centrifuging immediately before inserting each new portion of medium. If this was not done, the tubes were likely to become coated with precipitate, and the blanks to become unusually large.

Fig. 9 shows the typical results obtained in a test of this nature, and in Pl. 1 are photomicrographs showing the visible changes in the cultures. At the end of 120 hr., the cultures had spread to the limits of the plasma, and adjacent cultures were growing into one another. The central portions, which originally contained a kernel of compact heart tissue, were diffuse so that the core of each culture was translucent, and much larger than at the start. These visible changes were accompanied by a three-fold rise in RNAP, but once more there was hardly any increase in DNAP. The latter certainly rose slightly over 72–96 hr., but later when the diffusion of the original kernel of tissue was most noticeable, the DNAP fell. This behaviour was again reflected in the RNAP/DNAP ratio, which increases from 2·2 to 5·8.

The failure of the DNAP to increase was confirmed in another test, the results of which are
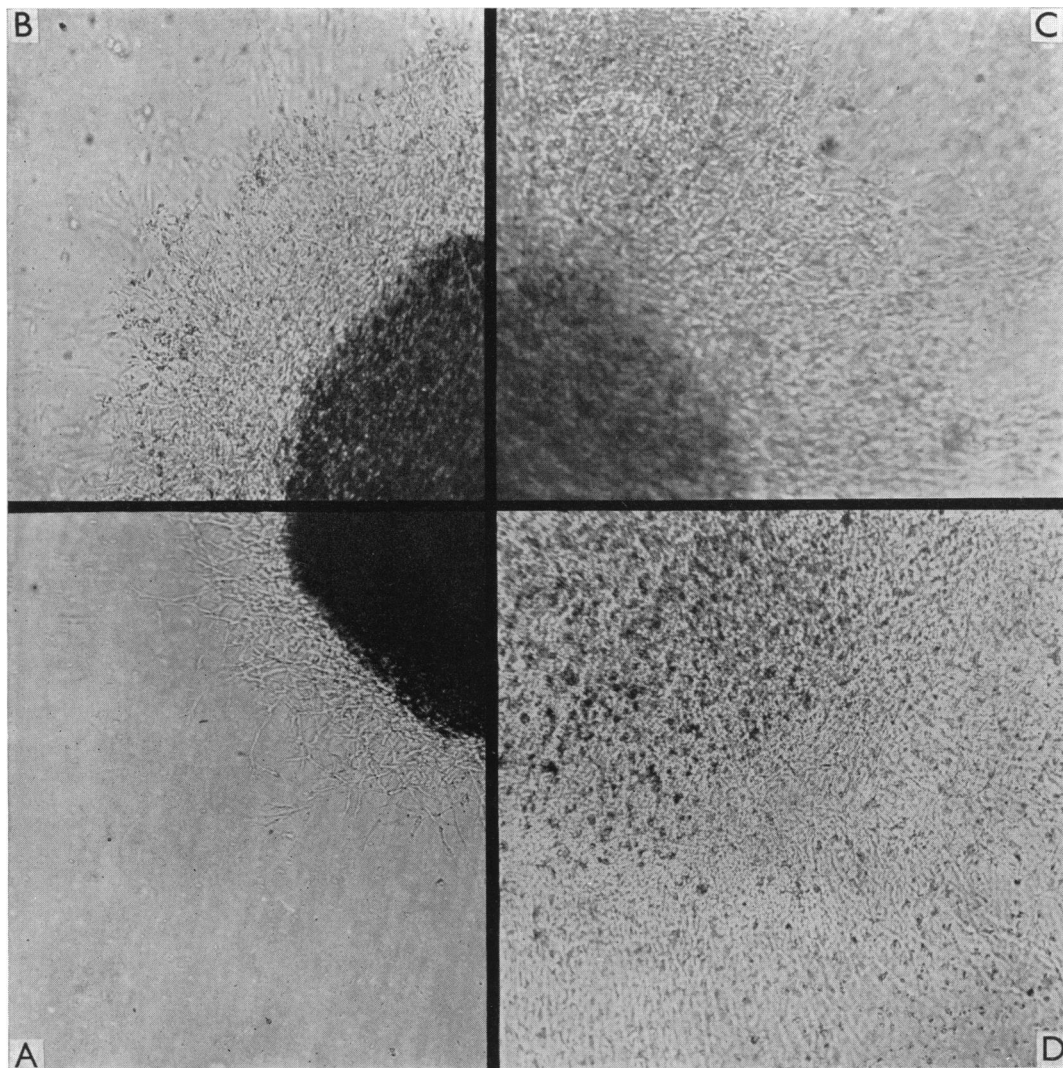
Photomicrographs of a single explant of 12-day chick-embryo heart at different stages of growth. A. Sector of explant after 2 days in plasma with Tyrode solution as fluid phase. Embryo extract-serum mixture (EES) added at this time. B. Same sector 24 hr. later. EES renewed again at this time. ·C. Same sector after further 24 hr. EES again renewed. D. Same sector after further 48 hr. Note progressive thinning out of the central zone as growth proceeds. Magnification, ×52.

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shown in Fig. 10. There is even a tendency for the DNAP to fall, particularly in the later stages when there was little change in the RNAP which reached its maximum at 96 hr. The RNAP remained at this high level for a further 72 hr., during which period growth (as observed by the area of the culture) had apparently stopped. However, the appearance of the cultures continued to alter, as a result of the thinning out of the central portions which became more diffuse and extensive.
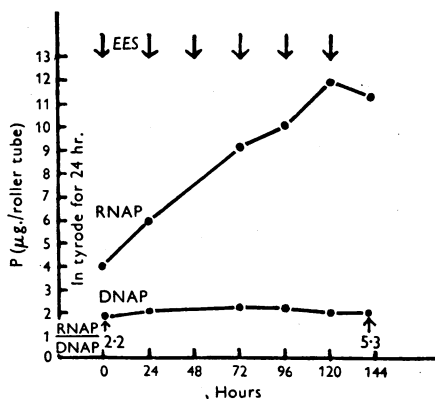

Fig. 9. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over 144 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows). See also collective legend for Figs. 2–8.
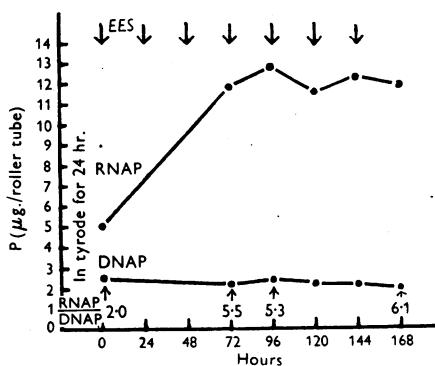

Fig. 10. Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over 168 hr. following the addition and renewal of EES at intervals of 24 hr. (as shown by arrows). See also collective legend for Figs. 2–8.

In these two tests, as in the others already described, the explants were cut relatively large and each roller tube was calculated to contain 10–11 mg. of fresh heart tissue at the start of the growth period. Under such conditions it has been confirmed that the rise in RNAP is of the order of 250–300 %, while the DNAP only rises in the early stages, at the most 25 % above the initial resting values. This rise

in DNAP is temporary and is followed after 96 hr. by a fall in spite of the continued high RNAP values which are found during the same period.

Effect of the size of cultures on their RNAP and DNAP changes during growth

A feature common to all the results so far described was our failure to obtain an appreciable and permanent increase in DNAP even in circumstances where growth of the cultures was apparently good. There seemed to be some link between the thinning out of the central portions of the cultures, and the tendency of the DNAP to fall while this was occurring. Such a fall in DNAP could be explained on the assumption that any increase in DNAP, by the production of new cells, was exceeded by its loss resulting from necrosis of the centre of the explants. This would occur when the tissue pieces were large enough to prevent the cells in the centre from receiving an adequate supply of metabolites from the nutrient, or from getting rid of the waste products of their resting metabolism. It was also possible that the amount of growth-promoting medium (0·5 ml. was the maximum which could be used in our roller tubes) was insufficient to provide for the continued existence of the relatively large cultures which we employed.

Support for these interpretations was available in the observations of Brues et al. (1944) on the growth of cultures of minced chick-embryo muscle in a peptone medium deficient in some of the factors thought to be necessary for growth. They showed that an uptake of P from the medium could be used to measure growth in cultures of chick muscle, and found that, in a peptone medium insufficient for tissue synthesis, cultures continued to grow at the periphery while losing weight by necrosis of the central portions. In tests using a medium fully adequate for growth, the cultures increased their P content until central necrosis balanced out or slightly exceeded growth at the periphery.

Our technique was accordingly modified with the object of reducing the initial size and weight of the tissue cultures to a minimum. This involved cutting the heart tissue into very small pieces, and planting them in rows of eight in 0·05 ml. of plasma, instead of 0·1 ml. as used previously. The weight of tissue per tube in the resting state was estimated to be about 2 mg. as compared with the 10–15 mg. used in earlier tests. This necessitated the pooling of material from two roller tubes in order to determine the amounts of RNAP and DNAP in the early stages of growth. Once the cultures had grown appreciably it was possible to carry out determinations on the contents of single tubes. In these tests, the RNAP was measured in amounts varying between 1·5 and 7·0 μg. P and the DNAP, in amounts between 0·5 and 1·0 μg. P. All points are the mean of two determinations, and as shown in Tables 1 and 2 the final corrected RNAP and DNAP increases were at least twice as large as the corresponding plasma blank increases.

The initial resting levels of RNAP and DNAP in the cultures themselves vary only slightly between 0·65–0·75 and 0·25–0·35 μg. P per roller tube, respectively. These

Table 1. *Comparison of changes in amount of ribonucleic acid phosphorus (RNAP)*
in tissue and plasma tubes

Test	Plasma/tube (ml.)	Hr. in contact with embryo extract (EE) or embryo extract-serum mixture (EES)	RNAP at 0 hr. (μg.)		RNAP increase at end of growth period (μg.)	
			Tissues*	Plasma	Tissues*	Plasma
40	0·3	48	6·43	0·45	+4·05	+0·43
41	0·3	11	4·65	0·26	+0·53	+0·21
42	0·3	36	5·71	0·26	+2·76	+0·71
46	0·3	24	3·12	0·40	+3·17	+0·02
49	0·3	58	3·32	0·16	+4·98	+0·56
50	0·3	120	3·98	0·12	+7·97	+0·48
51	0·3	168	4·94	0·10	+9·53	+1·29
53	0·3	48	5·74	0·12	+1·95	+0·39
57	0·3	49	2·64	0·12	+1·56	+0·01
Tests with smaller explants						
56	0·3†	120	1·28	0·18	+5·56	+0·50
58	0·3†	144	1·46	0·26	+5·29	+0·88
59	0·3†	144	1·18	—	+5·39	+1·58
62	0·3†	96 (EE)	1·55	0·04	+7·36	+3·28

* These are the figures obtained after correcting for plasma blank, and therefore the amounts represent the P gained or lost over and above that of the plasma blanks.
† Plasma/2 tubes.

Table 2. *Comparison of changes in amount of deoxyribonucleic acid phosphorus (DNAP)*
in tissue and plasma tubes

Test	Plasma/tube (ml.)	Hr. in contact with embryo extract (EE) or embryo extract-serum mixture (EES)	DNAP at 0 hr. (μg.)		DNAP increase at end of growth period (μg.)	
			Tissues*	Plasma	Tissues*	Plasma
40	0·3	48	2·41	0·19	+0·35	+0·04
41	0·3	11	1·85	0·06	−0·19	—
42	0·3	36	2·01	0·10	+0·04	−0·08
46	0·3	24	1·89	—	−0·12	+0·1
49	0·3	58	1·52	0·11	+0·24	+0·01
50	0·3	120	1·79	—	+0·22	+0·20
51	0·3	168	2·50	—	−0·68	+0·55
53	0·3	48	1·80	—	−0·28	+0·06
57	0·3	49	1·27	—	−0·28	+0·1
Tests with smaller explants						
56	0·3†	120	0·44	0·46	+0·88	−0·14
58	0·3†	144	0·60	0·20	+1·11	+0·36
59	0·3†	144	0·59	0·08	+0·67	+0·32
62	0·3†	96 (EE)	0·71	0·40	+1·1	+0·04

* See footnote, Table 1.   † Plasma/2 tubes.

cannot easily be further reduced since a lower limit is imposed on the size by the cutting and planting technique. With some practice it is possible to cut the tissues into pieces which have resting values close to 0·75 μg. RNAP and 0·35 μg. DNAP per roller tube. The same growth-promoting medium (EES) was employed, and each roller tube again received 0·5 ml. renewed every 24 hr.

With this modified technique substantial increases in the amounts of both RNAP and DNAP have been obtained. In Fig. 11 the RNAP is seen to rise steadily when determined at 48, 96 and 120 hr. On the other hand, there was a delay in the rise of the DNAP which showed little change at 48 hr. over its initial value, though by this time the area of the cultures had slightly increased. Later, when the cultures were very extensive and the core apparently larger than at the resting stage, an increase in DNAP was recorded. At 96 hr. the DNAP had risen by 80 % and at 120 hr. by 160 %; the corresponding RNAP increases were 243 and 353 %. The

ratio RNAP/DNAP, after rising to 7·4 at 48 hr., fell to 5·5 at 96 hr., and 5·2 at 120 hr., suggesting an approach to a new steady value. It also seems significant that this levelling out of the ratio occurs while the RNAP and DNAP are still increasing in amount.
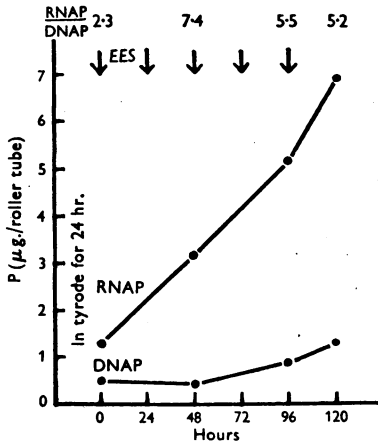

Fig. 11. Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 120 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows). Results based on 48 pieces of tissue from two roller tubes (see also collective legend for Figs. 2–8).
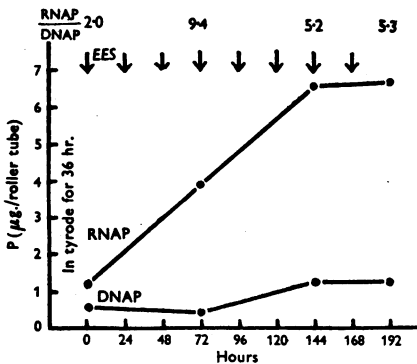

Fig. 12. Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 192 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows). Results on the same basis as Fig. 11.

Tests were run for longer periods in order to find the maximal values to which the RNAP and DNAP could rise under our experimental conditions. The results are shown in Fig. 12. Both RNAP and DNAP contents were maximal at 144 hr. after showing increases of 450 % for the RNAP, and of about 100 % for the DNAP, over the initial resting values. There was no change in the levels in the

subsequent 48 hr. The cultures did not appear to increase their area during this stage, but their centres appeared to spread out. This process was similar to the one occurring in the earlier tests, although there was now no tendency for the DNAP to fall at the same time.

Again in Fig. 12 the rise in DNAP did not occur until after 72 hr. and was, therefore, longer delayed as compared with the steady increase in RNAP obtained from at least as early as 48 hr. When maximal growth was reached the ratio RNAP/DNAP again levelled out at a value of 5·2. A final steady ratio of this order has been obtained in all the experiments in which substantial increases in both RNAP and DNAP were obtained. The other significant observation, which is a feature of all these tests, is the delay in the rise of DNAP for as long as 72 hr., even though the RNAP had increased appreciably in the same period.

Use of embryo extract (EE) as a growth-promoting medium

All the tests so far described have with one exception been carried out with EES as the growth-promoting medium. The preliminary tests, on which the decision to use this mixture had been based, had
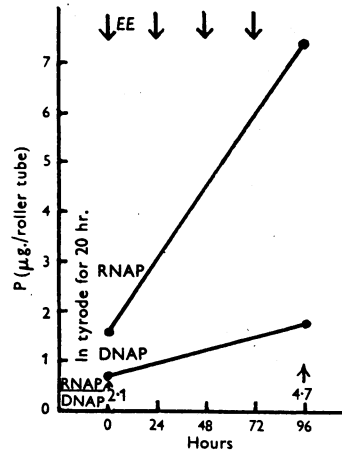

Fig. 13. Effect of replacing EES by embryo extract alone in maintaining growth of very small chick-heart explants *in vitro*. The changes in RNAP and DNAP are followed over 96 hr., the EE being renewed every 24 hr. (as shown by arrows). Results on the same basis as Fig. 11.

never shown an increase in DNAP. They had only shown that in certain circumstances large increases in RNAP were obtained when the medium was EES. It was necessary, therefore, to repeat the comparison of the two media using the modified technique.

In two tests in which EE was compared with EES of closely similar N content, there was an indication that EE was just as good a growth promoter as EES, but these results were not considered conclusive. Other tests, however, have shown clearly that EE alone is fully adequate as a growth-promoting medium. Fairly concentrated extracts with an N content of about 80 mg./100 ml. of extract have been used. As before, 0·5 ml. was allocated to each roller tube, and the EE removed every 24 hr.

In one such test, the results of which are given in Fig. 13, good growth occurred over a period of 96 hr. This was seen to be accompanied by a 155 % rise in DNAP and a 375 % rise in RNAP, comparing favourably with the corresponding increases of 160 and 355 % in a test in which EES was used (Fig. 11). It is concluded that EE alone as fluid phase is sufficient to supply all the necessary materials for growth of fibroblasts *in vitro*.

DISCUSSION

In analyzing the changes in RNAP and DNAP which occur in fresh explants growing *in vitro*, it is necessary to bear in mind that growth is not a simple process of multiplying cells, but a complex one in which the biosynthesis of cells at one point is accompanied by the breakdown of cells at another. Consequently any increases in either or both of the nucleic acids will be in proportion to new tissue synthesized in excess of tissue lost by the destruction of cells elsewhere.

In tests comparing the effects of embryo extract and Tyrode solution on the RNAP and DNAP content in the period immediately following planting, the fall in both, which leaves the RNAP/DNAP ratio unchanged, suggests that an intensive breakdown of damaged tissue is taking place. Within 20 hr. the cultures in Tyrode solution have reached their resting level, and subsequently the RNAP and DNAP will only fall slightly if at all. Embryo extract reduces the fall, but does not prevent it occurring; and, although the cultures increase in area in this period, it is evident that the breakdown of cells must greatly exceed the synthesis of new tissue. In the second 20 hr., the position is apparently reversed in both sets of cultures for the RNAP increases appreciably, while the DNAP increases slightly or remains unaltered.

The growing of fresh explants *in vitro* also involves the selective multiplication of one of the cell types, in this case the fibroblast of the connective tissue, affecting principally the ratio of RNAP to DNAP. In recent years a number of workers (Davidson & Waymouth, 1944 a, b; Schmidt & Thannhauser, 1945; Schneider, 1946) have shown that each tissue has its own particular content and ratio of RNA and DNA. These values will alter for any tissue according to its stage of development. For example,

there is a progressive fall in RNA, so that its concentration is appreciably lower in adult resting tissues than in developing tissues where a more intensive synthesis of protein is taking place (cf. Caspersson, 1947; Davidson & Waymouth, 1944 a, b; Davidson & Leslie, 1948). Tissues, such as pancreas and liver, which are actively engaged in the synthesis of protein in the organism, also have RNAP/DNAP ratios considerably higher than the ratios found in other body tissues (Davidson, 1947). Thorell (1947 a) has shown that the concentration of ribonucleic acid in haemopoietic cells falls from a value of 5 % in the cytoplasm and nucleolar apparatus of the early cells to less than 0·5 % in the mature cells. It would appear that this again involves a considerable drop in the RNA/DNA ratio from the exceptionally high values of the actively dividing cells.

For 12-day chick-embryo hearts a RNAP/DNAP ratio of between 2·5 and 3·0 has been confirmed (Davidson & Leslie, 1948). In the tissue cultures at the time of planting the ratio lies between 2·2 and 2·5, which may indicate a fall in RNAP perhaps due to washing out of ribonucleoprotein by the saline in which the tissue is immersed for cutting. There are, however, no precise figures for the nucleic acid content of connective tissue, nor have we any information about the RNAP/DNAP ratio in the actively dividing cells, except that it is likely to be appreciably greater than in the corresponding resting cell. Interpretations of our results in growing cultures have, therefore, to take into account changed RNAP/DNAP ratios produced by the increasing predominance of the fibroblasts, some of which are actively synthesizing protein during the process of division.

In all the tests in which relatively large pieces of tissue were used as explants, any increases in DNAP are small in comparison to the RNAP increases, and of a temporary character (Figs. 2–10). When the DNAP does rise about 20 % above its resting level it is usually after 48–72 hr. of growth; in later stages, it shows a tendency to fall slightly. At the peak of the DNAP curve, the RNAP is usually double its resting value, and the cultures appear to have grown considerably. The RNAP continues to rise until 120 hr., and thereafter remains unchanged, even though the cultures alter in appearance and the DNAP shows a fall. Over this period the thinning out of the central portions, and the falling DNAP content in conjunction with the high RNAP, suggests that the loss of material is slightly greater than the gain from the continuing production of cells. The cultures have evidently reached the maximal size which can be sustained under these particular conditions of growth.

The final RNAP/DNAP ratio in all these tests with relatively large cultures lies between 5 and 6— a considerable increase over the resting ratio of

about 2·2. These high values must represent a mean for the cultures, since all the cells are not likely to be dividing and carrying out protein synthesis with its associated high RNAP content. Consequently cells in the process of division may have a higher value for the ratio and resting cells a much lower ratio.

After our failure in these tests to obtain a substantial rise in DNAP, the possibility remained that growth of explants from chick-embryo heart involved the replacement of the original cells by new ones with a higher RNAP and a much lower DNAP content. This process could quite well have contributed to the results we obtained.

The relative importance of these different processes has been largely settled by the tests in which visible growth was accompanied by substantial increases in both RNAP and DNAP. Clearly, reducing the initial size of the cultures produces the conditions under which an increase of DNAP can be recorded, and although this is about half the corresponding increase in the RNAP, it does not follow that the content of DNAP per nucleus is also half the original. It is likely to be closer to its original value in view of the exceptionally high RNAP concentrations found in actively dividing cells (Thorell, 1947 a, b).

When suitably small explants are used, the RNAP rises five times above its initial value in 96 or 120 hr. This compares favourably with the two- to three-fold increase obtained in similar tests with larger cultures, and confirms the view that in the latter a loss of tissue at one point accompanies the increase of cells elsewhere. With the small pieces, once the maximum RNAP level has been reached, there is no tendency for it to fall.

Between 96 and 144 hr. the DNAP has increased to twice or more than twice its initial content, and, like the RNAP, remains at this new level when growth apparently stops. This behaviour of both RNAP and DNAP suggests that the cultures grow to a limiting size, determined by the environmental needs of the cells. The outer limits of the cultures are set by the boundaries of the plasma clot, and their proximity to one another. The core of each culture will not increase in extent once further growth prevents the proper nutrition of the cells in the centre.

It seems highly significant that, in all the tests in which growth has been studied over long periods, the final RNAP/DNAP ratio lies between 5 and 6. For the tests with small pieces the ratio is less variable and lies between 5·0 and 5·5. This confirms the view that the tissue cultures develop to an equilibrium state in which new cells are being produced at the same rate as others are being destroyed. It can also be said that the ratio of RNAP/DNAP in actively dividing fibroblasts is at least 5, and very probably greater than 5. Since this ratio is obtained while both RNAP and DNAP are increasing, and still

below their maximal values (Fig. 11), there can be no further selective multiplication of cells in this active growth phase, as this would involve a changing ratio as the dominant type of cell increased in number.

Another feature of interest in all our tests is the observation that the RNAP invariably rises appreciably for some time before there is any significant increase in DNAP. There are two possible explanations for these results. One seems likely to apply to the tests of short duration, in which changes were followed at 2-hourly intervals (Figs. 2–5). In these RNAP rises even when there is no visible sign of growth and no change, or a fall, in DNAP (e.g. Fig. 2). This behaviour strongly suggests that a synthesis of RNAP is occurring before additional DNAP is being produced by the appearance of new nuclei. As growth proceeds over longer periods another factor may come into play; it is possible that the new fibroblasts have a lower DNAP content than the average of 15 µg./100 mg. fresh wt. found in the original tissue. As a result, the RNAP could increase appreciably for some time, while the DNAP remained unchanged or even showed a slight fall. Such a process could quite well be occurring in tests of the type shown in Figs. 11 and 12. As late as 72 hr. after the start of growth there is apparently no increase in DNAP, although by this time good growth at the periphery can be seen with the naked eye. Very probably the situation is complicated by the continuous breakdown of other cells, in which case the DNAP content of the new type of cell may be only slightly below that of the original cells. An actual increase in DNAP would, therefore, occur when the alteration of cell type in the cultures was nearly complete.

A study of the biogenesis of the nucleic acids, and the possible conversion of one into the other during growth, is complicated by the occurrence in most tissues of at least two relevant synthetic processes occurring simultaneously. There is the conversion of RNA into DNA, which has been postulated to account for the decrease in the initial store of RNA during the period in which the DNA is increasing in the developing echinoderm egg (Brachet, 1933, 1937). Such a process implies the earlier synthesis of the RNA from nucleotides or other smaller components. As Brachet (1945) has pointed out with reference to the chick embryo, this synthesis of RNA from its precursors masks the possible conversion of RNA into DNA in studies on the growing tissues. There is, of course, a third possible synthesis, that of DNA from components which are not derived from RNA (Brachet, 1947), and recent work of Schmidt, Hecht & Thannhauser (1948) suggests that this might possibly take place in the early development of the echinoderm egg. They found, on measuring the changes in RNA and DNA, that the RNA remained constant while the DNA increased as much as tenfold during the first 24 hr. after fertilization. Their results do not, of course, exclude the possibility of conversion of RNA to DNA, but this could only occur if the turnover of RNA were shown to be very great during the rise

in DNA content. In phage-infected bacteria, Cohen (1947) has shown that DNA is in fact synthesized in the absence of significant turnover of RNAP.

Our evidence of increased RNAP before there is any production of cells, or change in DNAP, supports the view that cells in the process of division synthesize RNA and protein first of all, the synthesis of DNA following later (Brachet, 1945; Painter, 1945). This does not necessarily conflict with the views of Caspersson and coworkers (e.g. Caspersson, 1947; Thorell, 1947 a, b), who emphasize a different aspect of the process. Their evidence points to the nucleolus-associated chromatin (containing DNA) as the initiator of the synthesis of ribose polynucleotides (or RNA) and the associated proteins abundant in diamino-acids. These synthetic processes start at the nucleolus, and spread outwards to the nuclear membrane, and to the cytoplasm. These results imply a primary synthesis of RNA by the cell as a means of increasing its protein content before its actual division occurs. There is as yet no clear indication that an increase in DNA comes first, although the evidence of Cohen (1947) and of Schmidt et al. (1948) mentioned earlier can be interpreted in the particular cases considered as favouring the independent synthesis of DNA from components not derived from RNA.

The fluctuations of RNAP only seem to occur in the period which immediately follows the addition of growth-promoting medium. It is also evident that this is the time during which RNAP is increasing most rapidly, for in the later part of the 24 hr. period there is a definite fall in the rate at which it rises (Figs. 3 and 6). This suggests that in response to the stimulus of fresh growth-promoting medium, there is an initial intense synthesis of RNA, which slows down prior to a rise in DNA (Figs. 2, 4 and 6). However, it is difficult to account for the sharp fall in RNAP which was a regular occurrence and a feature of all the tests of this nature.

There are insufficient data on the changes in acid-soluble and lipid P to support any conclusions about their significance. The results of the test described earlier, however, suggest that the acid-soluble P in the extract and plasma is utilized during the period when increases in RNAP and lipid P are found in the growing tissues.

The experiments in which colchicine was added to the growth-promoting medium have clearly confirmed that we are measuring increases in both RNA and DNA as a result of their synthesis from materials present in the growth-promoting media. As there is a fall in both RNAP and DNAP with colchicine present, the increases with EES alone probably mask a simultaneous loss of material by the destruction of cells (Fig. 8).

Although embryo extract-serum mixture (EES) has been used for most of the tests, there is no reason to believe that different results would have been found with embryo extract alone. Once substantial increases in both RNA and DNA are obtained by the use of very small cultures, both EE and EES are equally good in producing this effect.

SUMMARY

1. Changes in ribonucleic acid phosphorus (RNAP) and in deoxyribonucleic acid phosphorus (DNAP) have been followed in chick-heart fibroblast cultures growing in roller tubes *in vitro*, and a study made of the conditions under which increases in both components can be obtained. By renewing the fluid phase with embryo extract (EE) or embryo extract-serum mixture (EES) every 24 hr., it has been possible to run tests of varying duration up to 7 days.

2. When relatively large explants were used (fresh weight of 24 pieces of tissues estimated as 10–15 mg.) only the RNAP content increased appreciably while visible growth was occurring. Over longer periods there was a relatively small and temporary increase in DNAP, although the RNAP continued to rise until it reached a steady level. In these tests the RNAP/DNAP ratio rose from initial values of 2·2–2·5 to final values between 5·0 and 6·0.

3. Appreciable and sustained increases in both RNAP and DNAP have been obtained when the cultures were grown from much smaller explants (fresh weight of 24 pieces of tissue estimated at 2 mg.). Under these conditions the production of new cells evidently exceeded the loss of tissue arising from central necrosis of the cultures.

4. The final RNAP/DNAP ratio in all tests of long duration has been between 5 and 6. This marked rise during active growth supports the view that protein synthesis is accompanied by increased RNA concentrations in the cells.

5. In tests of short duration with relatively large explants, a rise in RNAP always preceded a rise in DNAP, and this is considered to indicate that RNAP is a precursor of DNAP in the dividing cells.

6. The delayed rise in DNAP, while visible growth is occurring in tests using small explants, is suggested as evidence of the replacement of the original cells by fibroblasts having a lower DNAP content.

7. In one test in which acid-soluble P and lipid P were determined, the fall in the former in the tissue tubes suggests that this is incorporated in the nucleic acid P during the production of new tissue.

8. When colchicine was added to the extract, both the RNAP and DNAP fell in amount during the period when they would normally have increased or remained unchanged.

9. Embryo extract alone has been shown to be as adequate a medium for the growth of fibroblasts as a mixture of embryo extract and cockerel serum.

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The Irreversible Combination of Formaldehyde with Proteins

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The reaction of formaldehyde with proteins has been of interest for many years, owing to its industrial applications including tanning and plastics manufacture. Until recently the type of linkage formed was largely a subject for speculation rather than experiment. However, recent work has shed light on the details of the reaction. The literature has been adequately reviewed by French & Edsall (1945). Middlebrook & Phillips (1942, 1947) showed that at elevated temperatures the disulphide S of wool combines with formaldehyde, and that the reaction can be completely reversed by distillation with dilute phosphoric acid; it became apparent, however, that formaldehyde which combines with other groups cannot be completely removed in this way. Nitschmann, Hadorn & Lauener (1943) showed for casein that this method gave low results unless water was added, and a second distillation carried out; even so, the recovery of formaldehyde was not quantitative, and similar results were likewise obtained when 2N-sulphuric acid was used instead of dilute phosphoric acid. When the distillation was continued to dryness, the results were unreliable, owing to the destruction of the protein, and the production of bisulphite-binding substances.

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Wormell & Kaye (1945) and Fraenkel-Conrat, Cooper & Olcott (1945b) showed that the amide groups of proteins would combine with formaldehyde under acid conditions, and the latter authors found that the reaction was slow and greatly dependent upon formaldehyde concentration and reaction temperature. The present work was carried out to determine the effects of concentration of formaldehyde and pH on the amount of combined formaldehyde, which was stable to boiling dilute phosphoric acid and known as irreversibly combined formaldehyde.

METHODS

Materials

The wool used was a virgin Cape wool of about 64's quality, from which the tip ends had been removed, cleaned by successive extraction with ethanol, diethyl ether, and finally distilled water. Dark human hair was used for the supercontraction experiment. It was extracted by diethyl ether, any temporary set removed by allowing it to float freely in distilled water, and then carefully dried. Details of other protein preparations are as follows: casein, prepared from separated milk by precipitation with lactic acid (Van Slyke & Baker, 1918), $N = 15 \cdot 7 \%$, amide $N = 1 \cdot 58 \%$; collagen, sample from Dr D. Jordan Lloyd; edestin, sample from Roche Products, Ltd.; gelatin, Coignet 'Extra', amide $N = 0 \cdot 45 \%$; gliadin, sample from Prof. A. C. Chibnall; glycinin, prepared from fat-free soya meal by the method of