"NUCLEIC ACIDS IN GROWING TISSUES"

(A Study of the Biogenesis and Function of Nucleic Acids in Growing Tissues)

By

IAN LESLIE, B.Sc.

Thesis presented for the Degree of Doctor of Philosophy, The University of Glasgow.

MAY, 1951.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGMENTS

It is a pleasure to be able to record my gratitude to all who have helped me in the course of this work. I am especially grateful to Professor J.N. Davidson for the opportunity of carrying out this research, and for his constant encouragement and guidance in the past few years. I also wish to express my thanks for helpful advice to Dr. H.S.D. Garven and Mr. S.D. Morrison, Histological Division, Department of Physiology, to Dr. Iwo Lominski and Dr. A. O'Hea, Bacteriological Department, to Dr. H. N. Munro, Biochemistry Department and to Dr. E. Reid, Biochemistry Department, Cambridge.

Much of the work has been carried out with the technical assistance of Miss Margaret Meikle, and I am glad to express my appreciation of her conscientious and skilful work. In the actual preparation of this thesis, I have been greatly assisted by Mr. D.R.S. Cameron and Miss Pevie in the preparation of photographs, and I am also grateful to Mrs. Weatherstone, who typed the manuscript.

Finally, I should like to acknowledge the Imperial Chemical Industries Research Fellowship, which made it possible for me to carry out this research programme over the last three years.

INDEX.

SECTION I.

A. General Introduction.

While it is generally accepted that nucleic acids are in some way associated with protein synthesis and cell division, there remains unsolved the much larger problem of finding exactly where the nucleic acids play their vital part in the biochemical processes leading to peptide bond formation, to the construction of specific protein molecules, and to the reduplication of the genes or chromosomes in cell division. This search is still in its early stages, for it is only within the last ten years that reliable techniques have become available for the quantitative determination of nucleic acids and for the study of their metabolic activity in tissues. There is every reason to suppose that these will lead to an understanding of the metabolic relationships between the nucleic acids and other cell constituents, and it is hoped in the present review to make clear how far progress has already been made in this direction.

At the start it is essential to take into account the earlier confusion that existed concerning the character and distribution in nature of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Because the nucleic acid first extracted from yeast came to be accepted as plant

nucleic acid, while that extracted from calf thymus was thought to be characteristic of all animal tissues, it was possible for Jones in 1920 to write; "there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells, and the other from the nuclei of plant cells". It was this incorrect view of the distribution of the nucleic acids that led Le Breton and Schaeffer (1923) to propose that the important concept of a nucleo-plasmic ratio should be based on the chemical determination of purine N and total N $\left(\frac{\text{purine N} \times 100}{\text{total N} - \text{purine N}}\right)$ when, in fact, the purine of the nucleic acids was present in both the nucleus and cytoplasm of the cell. Again, for example, in the studies of Plimmer & Scott (1908) and Needham & Needham (1930) phospho-protein was determined from the amounts of inorganic phosphate obtained by incubation of tissue residue in dilute alkali at 37°, but it was not recognised at the time that RNA was hydrolysed by this treat-ment and appeared in the alkaline digest as organic phosphate. It can be assumed that the nucleoprotein P (NPP), as measured by their procedure, derived mainly from the DNA of the cells (cf. Schmidt & Thannhauser, 1945).

The work which ended this false distinction between animal and plant nucleic acids has been

reviewed in detail by Davidson & Waymouth (1944, 1944-1945). For the present it is sufficient to mention some of the decisive steps which led to our existing knowledge of the distribution of the nucleic acids. In 1924 Feulgen and Rossenbeck were able to show by means of the staining reaction specific for the deoxy-sugars that DNA was to be found in the nuclei of plant cells as well as in those of animal cells. About the same time there was growing evidence for the presence in animal tissues of pentose nucleic acids similar to the RNA isolated from yeast (Jorpes, 1924, 1928), and in 1933 Brachet postulated that the strongly basophilic cytoplasm and high content of purine N in oocytes, which apparently contained little DNA, was due to the presence of quantities of RNA. The view that RNA is largely located in the cytoplasm while DNA is confined to the nucleus of both animal and plant cells has been amply confirmed, and it seems to be established also for bacterial cells, in which there is a nuclear element containing DNA amongst the strongly basophilic cell substance (Tulasne & Vendrely, 1947; Davidson, 1950).

The development of techniques which could reveal changes in concentration of RNA and DNA

within the cytoplasm or nucleus, led to the association of nucleic acids with the processes of cellular growth and protein synthesis. With the aid of their spectrophotometric technique for measuring intensity of absorption of ultra-violet light between 240 and 300 mμ by purines and pyrimidines, Caspersson and his colleagues in Stockholm were able to determine the concentration of nucleotides at particular regions within the cell structure. Although this method does not directly distinguish RNA (or polyribonucleotides) from DNA, the latter was located within the nucleus by its selective staining in the Feulgen reaction, Brachet, in his histochemical studies, followed the changes in RNA concentration by the intensity of basophilic staining by toluidine blue or pyronine, and, by using ribonuclease to remove RNA from tissue sections, he was able to show convincingly that the basophilia of the cytoplasmic granules was indeed caused by the presence of RNA (Brachet, 1940).

Working independently and with these different techniques, Brachet (1941) and Caspersson (1940, 1941) concluded from their evidence that increased protein synthesis with the cell nucleus or cytoplasm was always accompanied by increased

concentration of nucleic acids. As early as 1938 Caspersson & Schultz were led by their observations on Drosophila chromosomes to suggest that "the nucleic acids are necessary prerequisites for the reproduction of genes, and they are probably necessary for the multiplication of self-producing molecules in general". It was later established that whenever the intense production of cytoplasmic proteins occurred, as in rapidly growing yeast cultures (Caspersson, 1936), in the dividing cells at the onion root tips (Caspersson & Schultz, 1939), in gland cells (Caspersson, Landstrom and Aquilonius, 1941), and in the tissue cells of the early chick embryo (Caspersson & Thorell, 1941), it was accompanied by intense cytoplasmic absorption by pentose nucleotides. While this work in its early stages laid more emphasis on protein synthesis during cellular growth, Brachet (1940) observed abundant RNA in the cytoplasm of the secretory cells of the pancreas, in the Nissl bodies of nerve cells, as well as in the cytoplasm of rapidly proliferating cells.

Subsequently, by the application of chemical methods for measuring total nucleic acid concentrations in tissues, Davidson & Waymouth (1944) were able to confirm that these were

generally higher in embryonic than in adult tissues. It also became clear that the ratio of RNA:DNA was greatest (3-4) in cells from tissues such as liver and pancreas, in which protein synthesis can be assumed to be intense (cf. Davidson, 1947); and in contrast, very much lower ratios (0.2-1.0) existed in the cells of spleen and thymus with smaller cytoplasmic volumes.

The conclusion that the nucleic acids are essential agents in protein synthesis then rests primarily on the observation of their abundance in cells in which protein synthesis is known to be intense. But it is important to know precisely how the nucleic acids are related quantitatively to protein formation in the cells. In an attempt to obtain this information, Caspersson has used his microspectrophotometric technique to determine the concentrations within the cells of proteins having either a high diamino-acid or high cyclic aminoacid content. There are, however, serious limitations to the accuracy of these measurements, particularly as applied to the changing concentrations of cells undergoing mitosis (Caspersson, 1950). What was observed in such studies was essentially a static picture of the cell in various stages of growth and activity, with the

result that the events, which linked the stages together, had to be deduced by the observer. With the introduction of reliable chemical methods for determining RNA and DNA in animal and plant tissues (Schmidt & Thannhauser, 1945; Schneider, 1945; and Ogur & Rosen, 1950), the work of finding how nucleic acids are related dynamically to protein synthesis has been considerably extended.

An approach to the problem from quite a different direction has been made in studies on the part played by energy-rich phosphate bonds in the synthesis of peptide linkages. It originated in the proposition of Lipmann (1945) that the energy requirements for this synthesis could well be met by the phosphate-bond energy of adenosine-triphosphate (ATP). Shortly after this, phosphorylation of benzoic acid was shown to be a probable step in the synthesis of hippuric acid (Cohen & McGilvery, 1946) and more recent work has demonstrated that the synthesis of the amide group in glutamine (Speck, 1949) and of the peptide bonds in glutathione (Johnson & Bloch, 1951) are dependent on the presence of ATP, ADP or the adenine mononucleotide. But, this and other evidence available is insufficient to establish that a phosphorylation process is necessarily involved in peptide bond formation (Johnson & Bloch,

1951; Linderstrøm- Lang, 1949), and no evidence has yet been brought forward to indicate the occurrence of energy rich phosphate bonds in the nucleic acid molecule.

The suggestion made some time ago by Ostern, Terszakowec & Hubl (1938) that, in yeast, RNA might act as a reservoir for the supply of nucleotides such as ATP, is still without experimental backing. It is perhaps of more significance that Speigelman & Kamen (1946) were able to detect an increased flow of labelled phosphate from the RNA fraction of yeast cells which were induced to synthesise protein. And Brachet (1947) drew attention to the simultaneously high concentrations in amphibian embryos of RNA, DNA and ATP in the dorsal half of the gastrula, when this was developing more rapidly than the ventral half. On the other hand, Chantrenne (1944) proposed that the nucleic acids, by reason of the insoluble complexes they form in combination with protein, may act in protein synthesis by precipitating newly formed protein molecules and so removing them from the site of the synthetic processes.

Before looking at the subject in greater detail, it is necessary to comment on the nomenclature of the nucleic acids. The earlier terms

zymo- or phytonucleic acid, and thymo- or chromonucleic acid have been superseded by the chemical names ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively. But the term pentose-nucleic acid is frequently used instead of RNA, since, strictly speaking, the identity of the pentose as ribose has been established only for cytoplasmic nucleic acid extracted from yeast (Gulland, 1943: Barker, Farrar and Gulland, 1947), from liver Davidson & Waymouth, 1944), from the tubercule bacillus (Vischer, & Chargaff, 1948), and from tobacco mosaic virus (Vischer, Zamenhof & Chargaff, 1949). Similarly, the name deoxypentose nucleic acid is often used instead of deoxyribonucleic acid, because the identity of D-2-deoxyribose has been definitely established only for the DNA of calf thymus (Levene & Mori, 1929), although the sugar in the DNA of spleen (Chargaff, Vischer, Doniger, Green & Misani, 1949), and of tubercule bacillus (Chargaff, Zamenhof & Vischer, 1949) has been shown to be chromatographically identical with that from thymus. However, for reasons of convenience, it would seem better to keep to the term deoxyribonucleic acid or DNA in describing the deoxypentose-polynucleotides, and to use the name ribonucleic acid or RNA in describing pentosepolynucleotides, as has been

suggested by Davidson (1950).

B. The Intracellular Distribution of Nucleic Acids and Enzymes.

We have seen that the general role of the nucleic acids in protein synthesis became apparent once it was known how RNA and DNA were distributed between cytoplasm and nucleus of the cell. It is now necessary to examine in much greater detail the intracellular distribution of RNA and DNA, since during the last twenty years it has become increasingly clear that within the cell, certain enzyme systems, at least, are localized or concentrated within definite cellular elements, such as the nucleus or mitochondria (cf. Bradfield, 1950).

Largely as a result of work of Caspersson and his colleagues on the ultra-violet absorption of mitotic chromosomes and of resting giant chromosomes of the Drosophila salivary gland, it has become accepted that DNA is one of the main structural components of the chromosomes. Along with the DNA, Caspersson (1941) also found evidence for the presence of basic proteins of the histone type, a higher protein characterized by its cyclic amino-acids, and a small amount of RNA. This general concept of chromosome composition has found support in the isolation cell nuclei of the non-histone protein, 'chromosomin' (Stedman & Stedman, 1943, 1947) and

the confirmation of the presence of such a protein in nuclei and nuclear fragments by Mirsky & Pollister (1947), Jeener (1947), and Stern (1947).

Evidence, based on the Feulgen reaction, for the presence of DNA in the chromosome structure has been questioned by Stedman & Stedman (1943, 1950). Their view, that DNA in combination with histone is primarily the substance of the nuclear sap, and that the developed Feulgen stain is selectively absorbed on chromosomal protein during the reaction, is strongly contested by a number of authors (Di Stefano, 1948; Stowell, 1947; Brachet, 1946, 1947; Barber & Callan, 1944; and Caspersson, 1949).

While claims have been made that RNA is also present in the chromosomes (Caspersson, 1941), and is to be found in the residual chromosomes, prepared by Mirsky and Ris (1947), there is little doubt that RNA in the nucleus is largely located within the nucleolar region of the resting cell. This was originally suggested by Caspersson & Schultz (1940) on the basis of ultra-violet absorption and negative Feulgen staining, and it was confirmed in histochemical studies by Brachet (1941), Gersh (1943) and Mitchell (1942); more recently Pollister and Leuchtenberger (1949) have suggested that a less highly polymerized form of RNA was to be found in the nucleolus. In

rat liver cells, Davidson & Waymouth (1946) reported that the ultra-violet absorption could not be entirely removed by ribonuclease, and although the central area of the nucleus did contain RNA, the periphery was distinguished by its DNA content. It seems certain that, here, the DNA corresponds to the nucleolus-associated chromatin of Caspersson (cf.1950).

The relatively large nucleoli of nerve cells have proved particularly suitable for the study of nucleolar composition, Hyden (1943) for instance, was able to show that an accumulation in the nucleolus of RNA and protein preceded the appearance of ribonucleotides in large amounts in the region of the nuclear membrane, and was apparently closely followed by the synthesis of cellular protein. This sequence of events was particularly noticeable in embryonal nerve cells during the period when the total protein in the cytoplasm increased more than 2000 times. From similar observations on the nucleolus in chick embryo cells (Caspersson & Thorell, 1941), in bone marrow cells (Thorell, 1944), and in tumour epithelial cells (Caspersson & Santesson, 1942), the Swedish workers have established the view that the onset of the processes leading to protein synthesis originates at the nucleolus.

Support for this concept of cellular organis-

ation has come from studies by Lagerstedt (1949) on changes in nucleolar size and composition in rat liver cells. By starving the animals or by keeping them on a low protein diet, he was able to observe a diminution in nucleolar size. A return to an adequate diet resulted in rapid enlargement of the nucleolus, followed by a concentration of basophilic cytoplasmic particles (containing RNA) round the nuclear membrane. As this occurred, the total nitrogen content of the liver began to rise.

More recently, evidence of a different nature has accumulated which might be accepted as additional support for the view that the nucleolus initiates RNA synthesis (and hence protein synthesis) within the cell. This evidence is based on studies of ^{32}P incorporation into the RNA of liver cells, but it is necessary to treat the results with some caution in cases where there has not been adequate purification of the RNA before its radio-activity was measured (Davidson, Frazer and Hutchison, 1951). This caveat applies to work by Marshak (1948) who first suggested that ^{32}P enters the cell most rapidly through an unknown labile polynucleotide, which was possibly RNA and certainly not DNA. However, with rather more reliable techniques, Barnum & Huseby (1950) found that ^{32}P was incorporated

into the nuclear RNA much more rapidly than into cytoplasmic RNA of mouse liver cells; for example, 45 minutes after injection of labelled phosphate, the nuclear RNA had a specific activity 200 times higher than the RNA of the microsomes (or small granules). Like Hevesy (1945) and Ada (1949), Barnum & Huseby showed that the situation was quite the reverse in the case of phospholipid, which incorporated ^{32}P most rapidly in the microsomes. In rat liver cells, too, Jeener & Szafarz (1950) were able to show that nuclear RNA had a specific activity 10 times higher than cytoplasmic RNA two hours after injection of ^{32}P as inorganic phosphate into the animals. As these last authors remark, their results support the view that "RNA is, <u>for an important part at least</u>, synthesised in the nucleus, passes into the region at the perimeter of the nucleus ..... (and) spreads into the rest of the cytoplasm."

Whatever the eventual fate of this hypothesis of intracellular organization, it is certain that the nucleus, far from being an inert carrier of genetic material, is the site of the most active RNA synthesis within the cell. Since there is specialisation of this nature within distinct regions of the cell, it is clearly important to know how nucleic acid metabolism

is related to enzyme distribution. The use of micro-techniques, developed by Linderstrøm-Lang and his colleagues in Copenhagen, have made it possible to determine the minute enzyme activity within cell and tissue sections (Linderstrøm-Lang, 1939), while microchemical methods have been used in the analysis of different cellular fractions, obtained by the controlled centrifugation of tissue homogenates. This second procedure was applied earlier to the isolation of mitochondria by Bensley & Hoerr (1934), of microsomes by Claude (1938, 1946) and to the isolation of nuclei by Dounce (1943, 1950) and Lan (1943).

It is not possible to discuss in detail in the present survey the great array of information on the location of intracellular enzymes which has been gathered by the application of these and other (cytochemical) methods, but it would perhaps be useful to summarise the position. The information on the intracellular location of enzymes, given on Table 1, has been collected mainly from reviews by Brachet (1950) Dounce (1950) and Bradfield (1950). It is, obviously, far from being a complete picture, but it makes clear that some of the enzyme systems, directly or closely involved in protein and purine metabolism, are to be found (a few of them, like

TABLE 1.

Intracellular Distribution of Enzymes

Tissues	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid
Rat liver	ARGINASE	Arginase		-
"	d-amino-acid oxidase	d-amino acid oxidase		-
"	Acid, alkal. phosphatases	Acid, alkal. phosphatases	Acid, alkal. phosphatases	-
	Dipeptidase	Cathepsin Peptidase	Dipeptidase	
Rat liver	URICASE			
Mouse liver	Esterase		Esterase	-
Rat liver	ATPase	ATPase	ATPase	-
Rat liver	-	CYCLOPHORASE	-	-
"	-	SUCCINOXIDASE		-
"	-	Cytochrome c	Cyt. c	
"	Cytochrome oxidase	Cytochrome oxidase		-
"	-	FATTY ACID OXIDASES	-	-
Rat liver	-		DPN CYT.c REDUCTASE	-
Rat liver, yeast	Catalase		Catalase	Catalase
	Lactic acid dehydrogenase			Lactic acid dehydrogenase
	-	-		GLYCOLYTIC SYSTEM
	-			Carboxylase

arginase, uricase, and alkaline phosphatase, in relatively high amounts) in the nuclei of liver cells. There may also be located in the nucleus a fairly high proportion of the adenosinetriphosphatase (ATPase) activity of the cell; on the other hand, the enzymes and carriers involved in the oxidative mechanisms of the cell are in some cases not detectable (e.g. cyclophorase, succinoxidase, and cytochrome c) although the presence in reasonably high activity of cytochrome oxidase, catalase, and lactic dehydrogenase has been reported (Dounce, 1950; Schneider, 1946; Chantrenne, 1947).

The large granule fraction (or mitochondria) seems to be unique in its enzyme equipment. Earlier work had shown that it contained enzymes for some of the linked reactions of the Krebs' tricarboxylic acid cycle (Schneider and Potter, 1949), and recently, Harman (1950) reported that the cyclophorase system was associated with cell mitochondria, and could not be detected in the nuclear or small granule fractions. Also present in the large granule fraction is the major part of the fatty acid oxidizing activity of rat liver (Kennedy & Lehninger, 1949), and certainly also some of the enzymes likely to be involved in protein metabolism. Because of this concentration of oxidative and synthetic enzyme capacity in the

large granules, Brachet (1945) has visualized them as ideal organelles for the synthesis of proteins within the cell.

Although the microsomes (or small granule fraction) are deficient in the complete oxidative enzyme systems, they are distinguished in adult cells by their relatively high RNA content, and by containing most of the DPN-cytochrome c reductase of the cytoplasm. In embryonic cells, where there is a shift in the preponderance of RNA towards the cell fluid (vide infra), the small granules acquire the major fraction of ATPase (Steinbach & Moog, 1945), at least, in chick embryo cells.

After prolonged high-speed centrifugation of tissue-homogenates, little of the enzyme activity related to protein, nucleic acid or oxidative metabolism can be detected in the supernate or cell sap. But this last fraction contains a good part, at least, of the glycolytic system, in addition to having a major share of the catalase, carboxylase, and lactic acid dehydrogenase activity of the cell. As will be seen later, the cell sap, although relatively deficient in enzymes, contains a RNA-lipoprotein complex, in which the RNA phosphate has a metabolic activity higher than that of RNA in other cytoplasmic fractions.

Quantitative information about enzyme distri-

bution has so far been obtained only by the cell fractionation techniques already mentioned. Nuclei, relatively free from cytoplasmic contamination, can be isolated from tissue homogenates suspended in citric acid, as in the method originally used by Crossman (1937), Stoneburg (1939) and Marshak (1941). While no definite rules can be made for centrifugation procedures, since tissue-homogenate characteristics are so widely different, the nuclear fraction is usually isolated by sedimentation at 600 g. for 5 minutes. It is even more difficult to draw hard and fast distinctions for the cytoplasmic elements. Mitochondria are, when rounded, particles about 2 μ in diameter, and are usually sedimentable (after separation of nuclear fraction) at forces of 19,000 g. for 10 minutes, or 3000 g. for 30 minutes from cytoplasmic homogenates in physiological saline or hypertonic (0.88 M) sucrose. It is safer, perhaps, to designate this the 'large granule' fraction of the cell. Microsomes, or submicroscopic particles of the cytoplasm, in the size range 50-200 mμ in diameter, are sedimented at 19,000 g. for 1-2 hours, and form the material of the 'small granule' fraction. The supernatant, which remains after such differential centrifugation, contains RNA-lipoprotein complexes (Gjessing, Floyd, and Chanutin, 1951), and can be conveniently described as the fraction

representing "cell fluid" or "cell sap".

The essentially arbitrary nature of this cytoplasmic fractionation is suggested by work of Chantrenne (1947) who separated mouse liver cytoplasm into five fractions by differential centrifugation. Their relative composition is shown on Table 2. The RNA per mg. N was highest in the fraction corresponding to the cell fluid, and least in the large granule fraction. Both lipid phosphorus and phosphatase were at maximal concentrations in the fraction which most likely corresponds in part to the small granule fraction, and apyrase (ATPase) was most concentrated in the large granule fraction of mouse liver.

In the majority of such studies by other authors, the cytoplasmic portion has been separated into three fractions, already distinguished for convenience as large granules, small granules and cell sap. Some of these have been summarized in Table 3, where results have been recalculated to show the distribution of cell components relative to the amounts found in the nuclear fraction. In contrast to the distribution on the basis of concentration per mg.N given above in Chantrenne's results, the values in Table 3 represent the relative amounts in the four fractions present in a constant weight of tissue.

The presence of DNA in the large and small

Table 2.

Relative concentration per mg. nitrogen in various fractions of mouse liver.

(Value of 10 assigned to concentrations in fraction A)

Cytoplasmic Fraction	RNA/mg. N	LP/mg. N	Alkaline Phosphatase/mg. N	Apyrase/mg. N
A (large granules)	10	10	10	10
B	18.1	7.7	14.0	8.5
C (small granules)	35.5	13.5	22.4	7.2
D	85	11.5	18.3	4.7
E (Cell fluid)	155	5.4	0.5	2.9

Based on the results of Chantrenne (1947).

granule fractions found in two cases (Price, Miller & Miller, 1948; Schneider, Hogeboom & Ross, 1950) suggests that not even the nuclei can be isolated intact from the rest of the cellular constituents. This danger of breaking down some of the particulate elements in the cells during separation is one of the main criticisms of the method, and cross-contamination of fractions must be taken into account when assessing results. Again, differences in technique seem to have produced differences in the distribution of RNA in the cytoplasmic fractions of rat liver (cf. Schneider & Potter, 1949; Schneider, Hogeboom & Ross, 1950; Price, Miller & Miller, 1948). All three groups agree that there is less RNA (20-50%) than DNA in the nuclear fraction, and that this amount represents only about 10% of the total amount of RNA in the cell.

There is rather better agreement between different groups with regard to the total N or protein distribution in cell fractions from rat and mouse liver (Schneider & Potter, 1949; Schneider & Hogeboom, 1950a,b; Price, Miller & Miller, 1948). A large part (40%) of the cellular nitrogen (or PN) was located in the cell fluid, while about 15% was to be found in the nuclear fraction; in contrast to its high RNA content, the small granule fraction had least N (but cf. Chantrenne, Table 2).

The presentation of the relative enzymic activity of the different cell fractions, on the basis of a constant value for the nuclear fraction, is made with one important reservation. According to Dounce (1950) certain enzymes, in particular, succinoxidase, (succinic dehydrogenase) are absent or present in extremely low concentrations in liver nuclei, and they may consequently appear in the nuclear fraction only as cytoplasmic contaminants. It may be assumed that, if the enzyme activity in this fraction represents less than 10% of the activity of the whole homogenate (as it does in the case of oxalacetic oxidase, succinoxidase, and DPN-cytochrome c reductase) the component in question is probably absent altogether from the nucleus. This view is strengthened by the fact that the nuclei contained 15% or more of the total N of the cell (Table 3). Any contamination would, moreover, come from intact cells and mitochondria, and would exaggerate the enzyme content of the nuclear fraction.

Riboflavin was largely associated with the large granules and the cell fluid (Price, Miller & Miller, 1948). Most of the cytochrome c (51%) was located in the large granule fraction, although only 64% of the total amount in the homogenate was recovered in the cell fractions (Schneider & Hoge-

Table 3.

Intracellular distribution of cell constituents in normal rat and mouse tissues. Proportions for the various fractions are based on the value of 10 assigned to amounts of DNA, total N or protein, and enzyme activity found in the nuclear fraction.

Tissue	Cell Constituent	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid	Homogenate	Author REF NO.
Rat Liver	DNAP	10	0	0	0	10	192
	RNAP	3.3	2.9	12.7	9.2	27.6	
Rat Liver	DNA	10	0.9			11.1	177
	RNA	2.1	8.5	8.7	8.3	31.0	
C3H Mouse Liver	DNAP	10	0.7	1.8	0	12.4	191
	RNAP	4.6	7.0	21.7	6.8	41.5	
Rat Liver	Total N	10	17.4	12.5	27.2	69	192
	Total N	10	17.4	14.8	33.9	76	190
	Protein	10	23.3	10.5	33.9	80	177
C3H Mouse Liver	Total N	10	13.1	12.9	21.0	56	189
Rat Liver	Riboflavin	10	78	20	51.5	156	177
	ATPase	10	10.4	1.6	-	-	188
	Cytochrome c	10	92	12.6	-	179	190
	Oxaloacetic Oxidase	10	42.5	0	4.8	96	192
Rat Kidney	Oxalacetic Oxidase	10	28.4	0	0	116	192
	Succinoxidase	10	66	14.1	-	119	190
Rat Liver	DPN-cytochrome c reductase	-	* 10	26.3	1.1	-	101
C3H Mouse	ATPase	10	16	4.9	1.6	32	191
	Succinoxidase	10	28.5	2.2	-	50.5	189
	Cytochromeoxidase	10	39.6	2.1	-	50.5	189
	DPN cytochrome c reductase	10	31.3	65.4	3.8	110	102

* No figure available for amount in nuclear fraction; value of 10 assigned to activity in large granules.

boom, 1950). As might be expected, the enzymes which showed a closely similar distribution to cytochrome c, were those associated with the cyclophorase system and the Kreb's cycle - namely, cytochrome oxidase, oxalacetic oxidase, and succinoxidase (Schneider & Potter, 1949; Schneider & Hogeboom, 1950 a,b). Adenosinetriphosphatase, from its relatively low amounts in the large and small granules, was clearly active in the nuclei as well as in the cytoplasm (Schneider, 1946; Schneider, Hogeboom and Ross, 1950). On the other hand, DPN-cytochrome c reductase was mostly located in the small granules, and it is unlikely to have been present in the nuclei (Hogeboom, 1949; Hogeboom & Schneider, 1950).

These various authors have reported similar studies on the intracellular organization of tumour tissues, and Table 4 summarizes the results obtained in work on mouse hepatoma 98/15, which arises spontaneously in the C3H mouse strain. Noteworthy amongst these was the higher proportion of RNA to DNA in the nuclear fraction of tumour cells (63%) as compared with that of normal mouse liver (46%), and the somewhat larger amounts of RNA and total N in the cell fluid of the hepatoma. There was, at the same time, nearly a 50% decrease in the ratio of cytoplasmic N : nuclear N the loss being greatest from the large granule fraction.

TABLE 4.

Relative amounts of constituents from various cell fractions of the cells from mouse hepatoma 93/15. (Value of 10 assigned to total content of nuclear fraction.)

Tissue	Cell Constituents	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid	Homogenate	Authors REF. NO.
C3H Mouse Liver	DNAP	10	0.7	1.8	0	10	191
	RNAP	4.6	7.0	21.7	6.8	41.5	
	Total N	10	13.1	12.9	21.0	56	191
Hepatoma 93/15	DNAP	10	0.4	1.8	0	10	191
	RNAP	6.3	4.1	15.3	10.1	38.5	
	Total N	10	5.7	8.6	20.2	43.9	191
C3H Mouse Liver	ATPase	10	16	4.9	1.6	32	191
	Succinoxidase	10	28.5	2.2	-	50.5	189
	Cytochrome oxidase	10	39.6	2.1	-	50.5	189
	DPN cytochrome c reductase	10	31.3	65.4	3.8	110	102
Hepatoma 93/15	ATPase	10	3.4	9.3	3.7	26.5	191
	Succinoxidase	10	34.8	4.5	-	59	189
	Cytochrome oxidase	10	49.5	12.6	0	73	189
	DPN cytochrome c reductase	10	18.4	35.1	5.3	67.5	102

A four-fold increase over the normal amount has also been reported for the RNA of the nuclear fraction of spleen from leukaemic mice (Arnesen, Goldsmith & Dulaney, 1949).

No great abnormality was apparent in the intracellular distribution of some enzymes in hepatoma tissue, apart from a fall in the relative amount of ATPase activity in the large granule fraction. All these observations, it must be remarked, refer to the relative amounts of enzyme activity in the various fractions, and give no indication of the differences per unit weight of tissue, or in total activity per cell between hepatoma and normal liver. Indeed, as the authors show and as will be discussed later, tumour tissue (weight for weight and per cell) shows distinct changes in enzymic content as compared with normal tissue (Schneider & Hogeboom, 1950 a,b; Schneider, Hogeboom & Ross, 1950).

Price, Miller, Miller & Weber (1950) have investigated intracellular changes during induction of liver tumours in rats fed on a diet containing the highly active carcinogen, 3'-methyl-4-dimethylaminoazobenzene (3'Me-DAB). In contrast to the mouse hepatoma, the cancerous liver tissue from these rats (Table 5) apparently showed no increase in the ratio RNA:DNA in the nuclei, and the proportion of RNA and riboflavin

Table 5.

Relative amounts of constituents in various cell fractions from livers of normal rats and rats fed on carcinogenic diet (3'Methyl-4-dimethylaminoazo-benzene.

Value of 10 assigned to DNA, protein and riboflavin contents of the nuclear fraction.

Tissue	Cell Constituents	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid	Homogenate
Normal rat liver	DNA	10	-	-	-	10
	RNA	3.7	8.7	9.3	6.1	29
	Protein	10	20.4	10	26.6	69
	Riboflavin	10	39.3	10	20	79
Rat liver on carcinogenic diet (3'Me DAB)	DNA	10	-	-	-	10
	RNA	3.1	1.1	2.5	3.5	11
	Protein	10	3.8	3.2	12.2	29
	Riboflavin	10	10	4.4	8.8	31

Based on the results of Price, Miller, and Weber (1950).

in the combined cytoplasmic fractions was very much less than in normal liver. Common to both induced and spontaneous tumours were relative increases of RNA and PN in the cell fluid, and a very distinct reduction of the proportion of PN in the large and small granule fractions.

From the work of Davidson (1945, 1947) and Campbell & Kosterlitz (1947) it has been established that liver cells lose cytoplasmic protein, RNA and phospholipid when rats are deprived of food or are kept on protein deficient diets, and, as already mentioned, Lagerstedt (1949) found a reduction in nucleolar size in similar circumstances. The results given by Muntwyler, Seifter and Harkness (1950 a) make it evident that the proportions of RNA and total N belonging to the large and small granules are decreased by a protein deficient diet, and that this is accompanied by a slight rise in RNA of the cell sap (Table 6). In a second paper, they draw attention to similarities in the intracellular pattern in cancerous and precancerous rat livers and in the livers of rats on protein-deficient diets (Seifter, Muntwyler & Harkness, 1950 b).

TABLE 6.

Yields in terms of constituents from various cell fractions of livers from control rats and rats kept on protein deficient diets.

(Value of 10 assigned to DNA and total N of nuclear fraction).

Tissue	Cell Constituent	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid	Homogenate
Controls on 3 week test	DNA	10	-	-	-	10
Liver from	RNA	4.5	1.6	15.5	11.3	31
	Total N	10	10.7	16.5	26.8	62
Liver from rats on protein-deficient diet for 3 weeks	DNA	10	-	-	-	10
	RNA	2.9	1.8	11.4	12.3	26
	TOTAL N	10	9.3	12.2	26.0	56
Liver from controls on 5 week test	DNA	10	-	-	-	10
	RNA	3.3	1.7	10.6	7.3	21
	TOTAL N	10	8.4	10.2	19.3	47
Liver from rats on protein-deficient diet for 5 weeks	DNA	10	-	-	-	10
	RNA	3.3	1.4	7.0	7.6	17
	TOTAL N	10	5.1	7.0	15.8	36

Results from Luntwyler, Seifter and Harkness (1950) J. Biol. Chem., 184, 131
Seifter, Luntwyler, and Harkness (1950) Proc. Soc. Exper. Biol. Med., 75, 46.

Brachet and his collaborators (cf. Brachet, 1950) have demonstrated interesting differences between young rapidly-proliferating cells and the more highly differentiated adult cells. Those from embryonic tissues showed less granulation of their cytoplasm than the adult cells, while a much higher proportion of their cytoplasmic RNA failed to sediment during ultra-centrifugation. Results of recent fractionation studies by Jeener & Szafarz (1950) can be seen in Table 7. There is a very distinct increase in the proportion of RNA found in the cell fluid in embryonic cells as compared with adult rat liver cells, and experiments with ^{32}P revealed that the specific activity of the RNAP in the small granules is lower than that in the large granules or cell fluid. This pattern differs from that found by the same authors in adult rat liver cytoplasm; here the RNAP of the large granules has the lowest activity (Table 7). This last observation is, however, not supported by Davidson, McIndoe & Smellie (1951), who have shown the specific activity of RNAP to be nearly as high in the large granules as in the cell sap of adult rat liver.

The evidence suggests that the pattern of the specific activities of RNAP amongst the fractions may be the same for adult and embryonic cells. Thus,

Table 7.

Relative amounts and specific activities of RNAP in the cytoplasmic fractions of cells from rat liver and mouse embryo. Values of 10 assigned to amounts in the large granules.

Tissue		Relative amounts RNAP (μg)			Relative specific activities		
		Large Granules	Small Granules	Cell Fluid	Large Granules	Small Granules	Cell Fluid
Rat Liver	1.	10	3.9	0.9	10	44.6	60
	2.	10	9.7	4.2	10	18.0	74
	3.	10	12.6	3.2	10	17.6	86
Mouse Embryo	1.	10	6.8	19.3	10	4.4	13.7
	2.	10	9.7	20.6	10	4.5	12.0
	3.	10	5.9	15.8	10	2.1	13.0

Based on results of Jeener and Szafarz (1950)

nuclear RNAP shows the greatest activity and the activities of RNAP of cell sap and large granules are rather lower but closely similar to one another; the lowest specific activity of all is found in the RNAP of the small granules. But, when glycine labelled with ^{15}N is incorporated into the protein of the various cell fractions, the situation is quite the reverse. Working with the livers of newly hatched chicks, Hultin (1950) has shown that ^{15}N-glycine was incorporated most rapidly into the small granules, and most slowly into the protein of the nuclear fraction. Although he did emphasise that the incorporation of glycine was not necessarily an indication of the intensity of protein synthesis, since "divergent amino-acid composition of a cell fraction may influence the isotope uptake" (cf.Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1949), he related the high activity of the microsomes to their relatively high RNA content. It remains, for the present, somewhat paradoxical that ^{32}P incorporation into RNA is lowest in this fraction, and greatest in the nuclear fraction, in complete contrast to the reversed rates of incorporation for ^{15}N-glycine into protein and for ^{32}P-phosphate into phospholipid.

Cellular changes during liver regeneration after hepatectomy have been studied by Price & Laird

(1950). The relative amounts of RNA and protein N in the nuclear fraction rose significantly over the period when cell proliferation was greatest, while the proportions of RNA increased sharply in the small granules (93%) and in the cell sap (125%) immediately prior to the onset of proliferation. From comparisons with intracellular changes in tumour tissues, Price & Laird concluded that "the relative amounts of protein N and pentosenucleic acid (or RNA) in the various fractions of the neoplastic cells studied, were quite different from either normal or regenerating liver". However, their results also show that the relative increases in nuclear RNA and PN, and in the RNA of cell sap and small granules of proliferating cells during liver regeneration, conform to the general picture already seen in embryonic and neoplastic tissues.

Summary

The available information on cell organization can best be summarized in terms of the four main cellular fractions.

(1) The nucleus contains the entire DNA of the cell, but only about 10% of the total RNA and 15% of the protein of the liver cell. Also found in high concentration in the nuclear fraction are enzymes involved in

purine and protein metabolism. On the other hand, many oxidative enzymes and carriers are absent, though cytochrome oxidase and ATPase have been detected in moderately high activities.

In rapidly proliferating cells, as in neoplastic and regenerating tissue, there is an increase in the proportion of RNA and PN in the nuclear fraction. Studies with labelled phosphate have all shown that the rate of incorporation of ^{32}P is much more rapid in nuclear RNA than in the RNA of other fractions, and the available evidence strongly suggests that RNA synthesis is initiated in the nucleolus.

(2) The large granule fraction has the lowest content of RNA of the three cytoplasmic fractions, but it reveals unique enzyme capacity in possessing the entire cyclophorase and fatty acid oxidation systems, as well as enzymes likely to be involved in protein synthesis.

The proportions of RNA and PN belonging to the large granules are reduced from the normal adult level in embryonic cells, in tumour cells and in liver cells depleted of protein during starvation. In both embryonic and adult cells the incorporation of ^{32}P into the RNA of this fraction is probably as rapid as it is into the RNA of the cell sap.

(3) The small granules (or microsomes) apparently contain most of the RNA of normal cells, though their

PN content is the lowest in the cytoplasmic fractions. They are distinguished by having most, if not all, of the DPN-cytochrome c reductase of the cell.

The RNA and PN of the small granules are diminished under the same conditions as cause their reduction in the large granules. While the incorporation of ^{32}P into RNA is slowest in this fraction, and greatest into the nuclear fraction, the situation is quite the reverse for the incorporation of ^{15}N-glycine into protein and of ^{32}P-phosphate into phospholipid. (4) The cell sap has the major share of the protein of the cell, but is almost entirely lacking in the enzyme systems involved in oxidative and synthetic processes. On the other hand, the bulk of the glycolytic capacity of the cell is found in the cell sap, along with such enzymes as catalase, carboxylase, and lactic acid dehydrogenase.

There are considerable increases above the normal adult level in the proportions of RNA and PN belonging to the cell sap in embryonic cells, in tumour cells, and in regenerating liver cells just prior to rapid proliferation. After nuclear RNA, the RNA of the cell sap shows the greatest incorporation of labelled phosphate in both adult and embryonic cells.

C. THE DNA CONTENT OF THE NUCLEUS

For a variety of reasons deoxyribonucleic acid has come to be closely associated with the hereditary characteristics of the chromosomes. As mentioned earlier, Caspersson and Schultz (1939) concluded that it was an essential element for protein synthesis in gene reduplication, and the same could now be said for its presence in viruses, such as neurovaccinia, the influenza virus, rabbit papilloma virus, and the Escherichia coli bacteriophage (cf. Hyden, 1947, Cohen, 1947).

An important aspect of this relationship between DNA and chromosomes lies in the discovery of its power to act as a 'mutagenic' agent in certain bacterial transformations. The transforming factors earlier isolated from Pneumococcus Types II, III, and IV (Avery, MacLeod, & McCarty, 1944)(McCarty & Avery, 1946) turned out to be specific forms of highly polymerized DNA, Like the factors found by Boivin and his colleagues (Boivin, 1947) to produce 'directed mutations' in E. coli strains, they lost their transforming power on treatment with deoxyribonucleic, but not on treatment with ribonuclease. The subsequent use of highly purified DNA in work with Pneumococci seems to have eliminated any possibility of the transformations being produced

by protein contaminants (Taylor, 1949). However, it has not been suggested that the DNA, per se, possesses specific genic activity; instead, the evidence available points "to the existence in nature of numerous deoxyribonucleic acids, differentiated by their particular biological qualities, and consequently also by some details in their chemical constitution" (Boivin, 1947).

Apart from this possibility of differences in chemical constitution between the DNA of one species and another (cf. Chargaff, 1950; Wyatt, 1951), there are two important repercussions of a biochemical nature. Although little is known about the direct interaction between genes and the general metabolism of the cell, it is supposed that the genes themselves are relatively stable agents which only rarely alter their structure. In addition, the substance of the chromosomes is normally reproduced only during mitotic activity. On these assumptions, the relatively slow incorporation of ^{32}P (as inorganic phosphate) and of ^{15}N (as labelled adenine) into the DNA of adult tissues led to the suggestion that DNA, by reason of its structural position in the genes, was not continously metabolised as are other cellular constituents. This view was reinforced by the discovery of the increased incorporation of ^{15}N-adenine into DNA during cell proliferation, and by the

tendency for ^{15}N to remain in the DNA although it was readily lost from RNA (Furst, Roll, & Brown, 1950). But serious doubts have arisen about this apparent stability of DNA, for when ^{15}N or ^{14}C was administered in glycine (Reichard, 1949; Elwyn & Sprinson, 1950; LePage & Heidelberger, 1951) or in the pyrimidine moiety of deoxyribosides (Reichard & Estborn, 1951), the incorporation of the isotope was seen to be as great in DNA as in RNA, or, at the very least, it was much greater than earlier work had suggested.

The important principle that DNA is constant in amount in nuclei of a single genotype, was first postulated by Boivin, Vendrely & Vendrely (1948). Their evidence for this was confined to vertebrates, but it indicated that the amount of DNA per nucleus was constant in the somatic cells in different adult tissues, and that the actual value was the same for all members of a single species. For the mammalian group, the 'constants' were of the order of 5-6 μμg. DNA per nucleus, but were distinctly lower (2-3μμg. DNA) in birds and fish. (Vendrely & Vendrely, 1950).

μμg. has been adopted throughout this section instead of the more conventional unit μg. x 10^{-6} (cf. Ogur et al, 1951)

Confirmation of this general relationship between DNA and genotype came from Mirsky & Ris (1949), who obtained, in some cases, values closely similar to those of the Vendrelys. There has since been a rapid extension of work on this problem, and considerable elaboration of the original concept. In Table 8 is summarized most of the existing information on the absolute amounts of DNA per nucleus (Ris & Mirsky, 1949; Davidson, Leslie, Smellie & Thomson, 1950; Davidson, Leslie & White, 1950; Davidson & McIndoe, 1949).

The general picture is in agreement with conventional genetic theory, which assumes that the chromosome complement is the same for most somatic cells in animals of one species. Where there is a definite departure from the diploid number of chromosomes, as, for example, in the haploid sperm cells, the DNA content is in fact about half the amount found in corresponding somatic cells (Table 8).

There are, however, exceptions. In liver tissue from man, cattle and rats, unusually high DNA values have been recorded by some authors (Table 8). In this connection, it must be mentioned that, as chemical estimations were made on known numbers of nuclei isolated by the citric acid method, the values given represent the average amount of DNA per nucleus

TABLE 8

DESOXYRIBONUCLEIC ACID CONTENT OF SINGLE NUCLEI ($\mu g. \times 10^6$)

Species	Liver	Erythrocyte	Kidney	Thymus	Pancreas	Sperm	Reference
Cattle	6.4		5.9	6.4	6.9		13, 213, 215
"	6.2, 8.4		6.3, 6.8	7.2		2.8	153
Calf	6.5						182
Pig	5.0		5.2				13, 213, 215
Guinea pig	5.9						13, 213, 215
Dog	5.0, 5.5		5.3				215
Man	5.8, 5.9, 6.3						215
"	10.0		7.5			3.4, 4.4	66
Rabbit	7.2						67
"	5.3						215
"	7–9		7–9				118
Rat	10.1–14.0						178
"	7–9		7–9				118
"	9.2–11.2						67
"	10.7		7.3				61
Mouse	7–9		7–9				118
"	6.0		5.0				215
Horse	5.8						215
Sheep	5.4, 6.1						215
Frog	15.7	15.0					153, 182
Green turtle	5.1	5.3					153
Fowl	2.4	2.3				1.26	153, 182
"	2.3	2.4	2.2		2.3		61
18-day chick embryo*	2.4						61
Duck	2.1	2.3					215
Goose		1.9					216
Turkey		1.9					216
Pheasant		1.7					216
Pigeon		2.0					216
Sparrow		1.9					216
Carp	2.8, 3.2						215, 216
"	3.3	3.5				1.64	153, 182
Shad	2.0	1.97				0.91	153, 182
Trout		5.79				2.67	153
"		4.8					216
Roach		7.3				3.7	153
Yellow tail	2.1						182
Red hind	2.1						182
Houndfish	2.2						182
Yellow grunt	1.2						182
Dusky shark	5.5						182
Tench		1.8					216
Eel		1.9					216
Pike		1.7					216

* Figures for the chick embryo (12) are as follows:

14-day: Brain, 2.2
Liver, 2.3

18-day: Heart, 2.2
Liver, 2.4
Muscle, 2.4

in a large population of cells. While this is the only accurate chemical method available for determining the absolute amounts per nucleus, it does not reveal variations in DNA content amongst individual members of the cell population. Such variations apparently occur in certain mammalian livers, in which can be found three distinct classes of nuclei in size ratios corresponding to those expected for diploid, tetraploid and octoploid cells (Jacobj, 1925; Biesele, 1944).

Recently Leuchtenberger, Vendrely & Vendrely (1951) have confirmed that differences in nuclear type are indeed responsible for the high average values mentioned above. By measuring DNA photometrically in single nuclei isolated from rat liver, they showed that their DNA contents fell into three distinct classes, corresponding to 1, 2 and 4 times the amount for normal somatic cells. Chemical estimations made on the same populations of nuclei gave the average values which would be expected for a mixture of the different nuclear type.

Another discrepancy has arisen with regard to the DNA content of Arbacia eggs. These are generally accepted as haploid, but when Vendrely & Vendrely (1950) measured the amount of DNA per cell by chemical means, they found the content was many times larger than that of the sperm cells. This is in general agreement

with earlier observations of Needham & Needham (1930) and of Schmidt, Hecht & Thannhauser (1948), who reported that the DNA in Arbacia sperm was about 3% of that in the egg. Whatever may be the reason for this, and it may be that the usual chemical methods cannot be applied without modification to this material, there is not the same discrepancy when measurements of DNA content are made on individual nuclei by spectrophotometric methods. Mirsky & Ris (1951) report that the egg of Ascaris megalocephalus has the same DNA content as the sperm when determinations are made on the Feulgen stained nuclei of egg and sperm, just after the latter has penetrated into the egg cytoplasm.

There is supporting evidence from an investigation on the DNA content of mouse oocytes.

Using a spectrophotometric method on Feulgen stained preparations, Alfert (1950) has demonstrated that the primary oocyte contains four times the amount of DNA in the final female pronuclei, and twice the amount present in the normal somatic cells. Here, again, the observed facts correspond more closely to the general theory, and are in agreement with similar observations made on spermatocytes and spermatids (Swift, 1950).

There are clearly a number of complicating factors which can affect the DNA content of the cell.

The view, based on cytological evidence, that chromosomes can be starved or overloaded with DNA according to circumstance (cf. Koller, 1947; Darlington, 1949) lacks real quantitative proof, and is contrary to all subsequent evidence (cf. Alfert, 1950). On the other hand, it is certain that during some stage of the mitotic process there must be a change in the DNA content of the cell, which, in rapidly proliferating tissues, could significantly alter the average amount of DNA per nucleus. The only evidence so far available on this aspect of the problem consists of the observation that in two chick embryo tissues at the 14th and 19th days of development, the average amount of DNA per nucleus is the same as in adult tissue (Davidson, Leslie, Smellie & Thomson, 1950).

Another instance, in which DNA demonstrates its biochemical stability, comes from studies on liver changes during starvation. Although rats on fasting lose considerable amounts of RNA, phospholipid and protein from the liver as a whole, the total DNA per liver remains unchanged (Davidson, 1947). Moreover, the average amount of DNA per nucleus remains the same as for liver cells from control animals (Davidson & McIndoe, 1949 and unpublished). For animals on a protein-deficient diet, Mandel & Mandel (1949, 1950) reported that the total DNA and the total number of

nuclei per liver were unaffected, although there were losses of other cell constituents.

Some information is now available on the DNA content of plant and bacterial cells. The distinctive feature of plant cells is their high DNA content. For corn root cells, Ogur & Rosen (1950) gave a value of 15 μμg. DNA per nucleus, and more recently they and their colleagues (Ogur, Erickson, Rosen, Sax & Holden, 1951) found values ranging from 50-370 μμg. DNA for nuclei of various anther cells of Lilium Longiflorum. Bacterial cells, on the other hand, show exceptionally low values for DNA; in Escherichia coli the average DNA is 0.01 μμg. DNA per cell (Boivin, Vendrely & Vendrely, 1948), and in Bact. lactis aerogenes constant values of approximately 0.02 μμg. DNA/cell have been reported for a wide variety of conditions of growth (Caldwell & Hinshelwood, 1950).

It is appropriate here to refer to one very interesting question which arises out of this work, namely, what is the significance of these constant, but highly specific, amounts of DNA found in nuclei of different animals and plants?. Vendrely & Vendrely (1950) presented one aspect of this problem when they discussed briefly the evolutionary significance of differences in the amounts of DNA per nucleus

in various species of fish and birds. Their point has been taken up at greater length by Mirsky & Ris (1951), who have extended the observations to invertebrates, as well as enlarging our information on vertebrates.

With the proviso that much work remains to be done, Mirsky & Ris (1951) put forward certain generalisations about the trend in the amounts of DNA per nucleus. In invertebrates it seems that the DNA content increases with the complexity of the organisms, being smallest in the sponges and largest in the squid. But there is not the same distinct trend amongst the vertebrates, where, if anything, evolutionary development is accompanied by a diminution in the DNA content of the nucleus. In fishes, for example, there are great variations from one species to another, but the DNA content per nucleus is of the same order for those in the same family group.

If the amount of DNA per nucleus has, as it seems, some significance in evolutionary development, there is still the question of the relationship of DNA to the chromosomes and to cell structure and function. Again the evidence is fragmentary, although Mirsky & Ris (1951) find a direct relationship between the DNA content of cells and cell mass in the homologous tissues from widely different species. This, of course,

recalls the concept, established by Boveri (cf. 1925) and Hertwig (1934;1939), that an increase in chromosome number in cell nuclei of one species would necessarily result in increased cell size. However, the relationship between DNA and cell size does not appear to hold for different cells within the testis of the pentatomid insect, Arvelius albopunctatus (Schrader & Leuchtenberger, 1950). Here, at least, a rather different mechanism is in operation, and the situation is best summarized in the authors' own words. "While the increase of nuclear, nucleolar and cytoplasmic volume seemed to be intimately linked with each other and correlated with an increase in protein and ribosenucleic acid, their quantitative changes were in no way reflected in any alteration of the amount of DNA in the nucleus."

It is necessary to consider now the DNA content of pathological cells. So far, the relatively few observations which have been made, concern the DNA content of tumour cells. In some cases there is apparently no difference between the DNA content of these cells and that of comparable normal cells. For example, the DNA content of rat hepatoma and cholia-ngioma nuclei is reported to be the same as in normal rat liver cells (Mark & Ris, 1949); nor was any change recorded in the DNA per nucleus of liver

cells during the induction of tumours with acetyl-aminofluorene or the azobenzene dyes (Cunningham, Griffin & Luck, 1950; Price, Miller, Miller, & Weber, 1950). Where a change has occurred in the nuclear content of DNA in tumour cells, it has in each case involved an increase in the average amount to twice the normal value. This applies to the GRCH 15 sarcoma of the fowl, where the amount per nucleus was 5.1 μμg. DNA (Davidson & McIndoe, 1949). Again, Klein & Klein (1950) reported a value of 14.2 μμg. DNA per nucleus for the Ehrlich ascites tumour cells; this is about twice the normal value (5-8 μμg. per nucleus) for mouse cells.

Although these results seem to suggest that the chromosome number is doubled in certain types of tumour cells, Klein, Kurnick & Klein (1950) consider that the increased DNA is connected rather with rapid cellular proliferation. There is, however, some support for the former view in the earlier observation that the DNA per mg. dry weight of isolated nuclei was unchanged in hyperplastic cells, but was increased twofold in chromosomes isolated from squamous cell carcinoma (Gopal-Ayengar & Cowdry, 1947). It is also significant that Klein & Klein (1950) found a value (7.0 μμg. DNA per nucleus) within the normal range for the ascitic tumour cells of the Krebs sarcoma.

The discussion up to this point has been mainly confined to the results obtained by biochemical methods of determining the DNA content of nuclei. Something must be said now of the important investigations which have been made with the spectrophotometric technique. Its particular merit is that it enables measurements of DNA content to be made on selected individual nuclei stained with the Feulgen reagent or methyl green (cf. Pollister & Ris, 1947). Its limitations lie in the considerable technical difficulties involved in photometric determinations within so complex a structure as the cell nucleus. Only a few brief comments are possible here. Both Mirsky & Ris (1949) and Swift (1950b) have stressed the importance of confining the determinations to nuclei in which there is an even distribution of the dyed material. Where this limitation has not been applied, as in studies of Schrader & Leuchtenberger (1949) and of Pasteels & Lison (1950 a,b,c, 1951), the conclusions reached have not been in accordance with those of other workers. It is also unfortunate that the absolute amounts of DNA per nucleus cannot readily be determined by this method.

In general terms, all the studies on the DNA content of selected individual nuclei have confirmed the view that the amount of DNA per

nucleus is decided by its particular complement of chromosomes. This was the case for cells from different strains of corn (Zea mays) examined by Swift (1950a), and the relationship was further emphasised by the step-like occurrence (2,4,8,16 or 32 fold) of increases in DNA in different classes of plant nuclei. In many animal tissues Swift confirmed the uniform character of their nuclei, which he graded as Class I (i.e. diploid in character). In others, such as liver, pancreas, thymus, blood lymphocytes and Sertoli cells, he found some nuclei with twice (Class II) or four times (Class III) the average amount of the Class I group. Although Pasteels & Lison (1950 a) also obtained three grades of nuclei in the liver and pancreas of adult rats, their lowest group contained less DNA than the amount associated with normal somatic cells. Their conclusion that these low values represent partial chromosome reduction in differentiating tissues is not supported by the work of Leuchtenberger, Vendrely & Vendrely (1951).

In their extensive studies on changes in DNA in the nucleus during the mitotic cycle, Pasteels & Lison (1950 b,c; 1951) have reached conclusions which are radically different from those adopted by other authors. For example, Pollister & Ris (1947) were led in their early photometric studies to

conclude that the "DNA increases in prophase to reach in metaphase about twice the amount of interphase nuclei". This is also implicit in the results of Swift (1950b), although he recorded that all the DNA values in early prophase fell into Class II, thereby suggesting that the increase from the Class I (diploid) level occurred during the interphase period. Finally, during mitosis of microspores in the anthers of Lilium Longiflorum, Ogur & Erickson (1950) and Ogur et al. (1951) were able to show by microchemical methods that the DNA doubles rapidly just prior to cell division.

While allowance must be made for possible differences in the cycle of events from one cell type to another, the process suggested by Pasteels & Lison does seem to be fundamentally different from the one envisaged above. From their observations on cells of the crypts of Lieberkühn, and on chick heart fibroblasts growing in vitro, they assert that the normal somatic DNA content is halved during mitosis, and is reconstituted rapidly during telophase to produce daughter cells with the original interphase amount of DNA. They state their position even more precisely with respect to mitotic activity in the early sea urchin embryo (Pasteels & Lison, 1951); "Donc, alors que le cycle mitotique s'étend sur une durée

totale de 40 minutes, la synthèse en DNA rétablit la valeur initiale du noyau (et la dépasse même) pendant une période inférieure à cinq minutes; cette période sa place immédiatement après la télophase, au cours du gonflement du noyau." Though they claim that their observations agree (in some respects) with those of Swift (1950), there is the vital difference that, on their own interpretation, the normal interphase content of DNA is halved, not doubled, during the course of the mitotic cycle.

They introduce a second new element into the discussion. Although many of their results would imply a strict genetic relationship for DNA in adult cells, they find consistently higher average values for the DNA of interphase nuclei in embryonic cells, and in rapidly proliferating cells. This was the case for ovarian follicular cells, in rats, and it was particularly evident during the rapid differentiation of cells in the sea urchin embryo. If the normal DNA content is actually doubled during mitosis, these results are quite consistent with the view that it is an integral part of chromosome structure. But, if the conclusion of Pasteels & Lison is correct, and the normal DNA content is halved during mitosis, the amount associated with the chromosomes must then be elevated according to the intensity of their

reduplication. This question can only finally be settled by future research, but it can be said that this new conception of the role of DNA is not supported by Swift (1950b), whose observations on embryonic nuclei showed that their DNA content fell into the same two classes found in corresponding adult cells.

SUMMARY.

The following are some of the important consequences of the integral part which has now been established for DNA in chromosome structure.

(1) Studies on the nucleotide composition of DNA, and on the transforming action on bacteria of DNA isolated from different strains, indicate that DNA composition varies from species to species.

(2) Although DNA shows high biochemical stability with regard to its incorporation of labelled phosphate and adenine, this is not supported by its relatively rapid incorporation of labelled glycine and pyrimidine deoxyribosides.

(3) The amount of DNA is constant in nuclei of a single genotype. The average amounts are established for somatic cells in different adult and embryonic tissues, and are the same for all members of a single species. For various organisms the amounts

of DNA per nucleus (or cell) are, roughly, as follows: bacteria, 0.01-0.02 μμg; birds and fish, 2-3 μμg.; mammals, 5-7 μμg.; frog, 15 μμg.; and plant cells, 15-370 μμg.

(4) Spermatozoa each contain about half the amount of DNA characteristic of the somatic cells. Spermatocytes and primary (mouse) oocytes have twice the amount found in the somatic cells.

(5) Certain mammalian tissues (e.g. liver, pancreas) contain a proportion of tetraploid and octoploid nuclei. Individual nuclei in these tissues show correspondingly high amounts of DNA, and the average value for a population of isolated nuclei is higher, as would be expected from a mixture of diploid and polyploid nuclei.

(6) In tumour cells, the available information shows that the average amount of DNA per nucleus is either the same as in the normal somatic cells (e.g. hepatoma, Kreb's tumour ascites), or twice that amount (GRCH 15 sarcoma, Ehrlich ascites).

(7) Confirmation of these results (which have been obtained by chemical determinations on large populations of isolated nuclei) has come from photometric studies on individual selected nuclei.

(8) Most of the evidence shows that the DNA per nucleus is doubled before metaphase, and that the

normal somatic amount is restored on cell division. Some evidence, which is not in accordance with this view and is still unconfirmed, has been discussed.

SECTION II.

EXPERIMENTAL

The research, now to be described, was designed to supply information on kinetic relationships between the individual nucleic acids (RNA, DNA) and other cellular constituents. It seemed particularly important to find precisely how protein synthesis and accumulation in growing tissues depended upon the accompanying variations in nucleic acid content; in addition, in some of the work it became possible to follow, simultaneously, the behaviour of two other phosphorus fractions, the acid-soluble phosphates and phospholipids. These studies fall into three sections;

(1) tissue culture studies using chick heart explants growing in roller tubes.

(2) the study of changing cellular composition of brain, heart, liver and muscle in the developing chick embryo.

(3) an investigation of the cellular content of RNA and DNA in normal and pathological human tissues.

A. CHEMICAL METHODS

In all three investigations, essentially the same method was used for the separation and determination of the various cell constituents. When tissue was collected, it was either kept frozen hard in solid

carbon dioxide until required, or immediately homogenized in a Potter-Elvehjem homogenizer or Waring blender with ice jacket in the presence of ice-cold 10% trichloracetic acid (TCA) and transferred to a 15 ml. centrifuge tube. The amounts used in the subsequent extractions depended, of course, on the initial mass of fresh tissue. In the chick embryo work, the weights of fresh samples ranged from 0.2-1.0 g., and 5 ml. volumes of reagents were used throughout the extraction of acid-soluble phosphorus (ASP) and phospholipid (LP); the number of extractions at each stage was also increased with the largest samples. For the smaller amounts in the tissue culture samples and in biopsy material from human bone marrow and liver, 2 ml. volumes were generally sufficient.

Material precipitated from the homogenate by TCA was centrifuged down at 2000-3000 r.p.m. for five minutes, and washed three to five times with a suitable volume (2-5 ml.) of 10% TCA, the washings being collected and added to the original acid extract, when required for the determination of ASP. To avoid any possibility of hydrolysis of RNA during acid extraction of the tissue, these operations were carried through in the shortest possible time, all material being kept ice-cold during manipulation; whenever possible, a refrigerated centrifuge was employed.

Extraction of lipid material was begun by resuspension of the tissue residue in 2-5 ml. of 80% ethanol, and continued as before by subsequent extractions with 100% ethanol, and with ethanol-chloroform (3:1) mixture at 80° on a hot air-bath for 30 minutes; this last extraction was repeated two to four times, and the tissue residue was finally dried after a single washing with redistilled ether. These extracts were pooled for the determination of total LP.

The determination of RNA and DNA in terms of their phosphorus content was based on the procedure developed by Schmidt & Thannhauser (1945). The dry residue was incubated overnight at 37° in N NaOH, 1 ml. of alkali being used for each original 100 mg. of fresh tissue. A suitable portion (from one-half to one-thirtieth) of the alkaline digest was taken for the determination of residual nitrogen (RN), and the remainder (or a suitable fraction of it) was neutralized with 2.5 N HCl and acidified with 30% TCA in sufficient amount to bring the final TCA concentration to 10%. This operation was carried out at 0°, and resulted in the precipitation of DNA and protein, the ribonucleotides from the hydrolysed RNA remaining in the acid solution. The precipitate containing the DNA was collected by centrifuging at 3000 r.p.m. for 10 minutes, and was freed from traces of RNA by two

washings with 1 ml. portions of ice-cold 5% TCA. The acid extract and the two washings were combined to give the RNA fraction.

The determination of RNA phosphorus (RNAP) and DNA phosphorus (DNAP) was based on the total phosphorus found in each of these two fractions. The RNA fraction contains, in addition to ribonucleotide P, a small amount of inorganic phosphate derived from phosphoprotein (cf.Plimmer & Scott, 1908), but this phosphoprotein P has been shown to be present only in minute amounts in most animal tissues (Schmidt & Thannhauser, 1945; Davidson, Frazer & Hutchison, 1951), and it has been disregarded in the present study.

I. Phosphate estimations.

Two different methods were used in the determination of total phosphorus present in the various fractions. For amounts between 10 and 200 µg. P, modifications of the method of Allen (1940) were employed.

(a) Reagents: 10 N sulphuric acid
100 vol. M.A.R. hydrogen peroxide, or
60% perchloric acid (PAC).
Amidol solution (1 g. amidol and 20 g. sodium metabisulphite in 100 solution which was kept in dark stoppered bottle).
8.3% ammonium molybdate in N/100 sulphuric acid.

(b) For amounts of P between 40-200 µg. The organic material was oxidised by heating with 1.2 ml. 10 N sulphuric acid, a few drops of 100 vol. M.A.R. hydrogen peroxide being used to complete the process. The volume of the cold digest was made up to 7.0 ml. with water and to this were added in turn, 2 ml. amidol reagent and 1 ml. ammonium molybdate reagent. After making the final volume up to 25 ml. with water and allowing the colour to develop for about 5 minutes, readings of colour intensity were taken in the Hilger Spekker absorptiometer, using an Ilford red filter (No.608). The final results, after correction for reagent blanks, were obtained from a calibration curve prepared for known amounts of inorganic phosphate. All results were expressed in terms of ug. phosphorus.

(b) For amounts of P between 10-40 µg. The organic material was oxidised by prolonged heating with 0.3-0.6 ml. 60% perchloric acid (PCA) at 210° with 1 ml. water for 15 minutes to hydrolyse polyphosphates, and, when cool, washed quantitatively with two 0.5 ml. volumes of water into 5 ml. graduated tubes. After addition of 0.4 ml. amidol reagent and 0.2 ml. 8.3% ammonium molybdate, the volume was made up to 5 ml. with water. The blue colour was allowed to develop completely and readings taken, as before, in the Hilger Spekker absorptiometer. The final results were corrected for

reagent blanks, and obtained as μg. P from a calibration curve prepared for this range of determinations.

(c) For amounts less than 10 μg. In the studies on tissue culture and biopsy material the amounts of phosphorus encountered in the various fractions was usually less than 10 μg. Here determinations were based on the method of Berenblum & Chain (1938) as modified by Davidson & Waymouth (1943). By this means it was possible to detect changes in the amount of P of the order of 0.1 μg. over the range of 1-9 μg. P.

Reagents. 60% perchloric acid (AnalaR).

5% ammonium molybdate dissolved in N/100 sulphuric acid.

Redistilled iso-butanol.

N sulphuric acid.

Dilute stannous chloride in N sulphuric acid (contains 0.5 ml. 40% $SnCl_2$ in conc. HCl per 100 ml. N sulphuric acid).

Ethanol-iso-butanol mixture (1:1).

Procedure. Excess liquid in the various tissue fractions was evaporated on hot air or water-baths, the organic material then oxidised with 0.3 ml. 60% PCA by prolonged heating at 210°, and, after heating at 100° for 15 mins. following addition of 0.5 ml. water, the final solution of inorganic phosphate was transferred to special mixing pipettes (Davidson & Waymouth, 1943). With the acid

solution and two washings of 0.5 ml. water, the volume in the pipette, before the addition of reagents, was 1.5 ml. To this was added, with mixing, 0.25 ml. 5% ammonium molybdate, and 1 ml. redistilled isobutanol. At this stage the reagents were thoroughly mixed and the layers allowed to separate completely before removing the aqueous phase containing excess molybdate. The iso-butanol layer was washed twice with 1 ml. volumes of N sulphuric acid, and the blue colour developed with 1.5 ml. dilute stannous chloride solution. In series of 6-14 samples, this last stage had to be done methodically, so that the atannous chloride solution was mixed and in contact with the isobutanol layer for the same time in each case. The blue iso-butanol phase, which remained in the pipette was transferred quantitatively to small graduated tubes, and with 1-1.5 ml. washings of ethanol-isobutanol (1:1), was made up to 5 ml. Readings were taken with a Hilger Spekker absorptiometer, using a red Ilford (no.608) filter, and a calibration curve prepared for inorganic phosphates standards. Here, again, it was important to correct for blank determinations made on the reagents used in the tissue extractions.

II. Nitrogen estimations.

Nitrogen was determined by the micro-Kjeldahl method using the distillation technique of Ma & Zuazaga

(1942). Preliminary digestion of the samples was carried out with 1 ml. nitrogen-free sulphuric acid and $CuSO_4$-K_2SO_4-SeO_2 mixture, or with 0.5 ml. 60% PCA, the two methods giving comparable results over the range of 100-250 µg. N. Particular care had to be taken with the PCA oxidation to avoid losses due to the decomposition of ammonium perchlorate (cf. Ogur & Rosen, 1950); this involved prolonged heating (8-12 hours) of the material in an air-bath at 210° in Pyrex boiling tubes covered with glass bulb condensers to retain PCA fumes. Occasional faulty batches of PCA giving low results, were discarded. The accuracy of both methods of digestion, as determined by estimates on standard solutions of ammonium sulphate, was - 2%.

The results of such determinations, made on appropriate portions of the alkaline digest, represented the amount of residual nitrogen (RN) in the sample. This, of course, included the nitrogen of the nucleic acids, and the figures for protein nitrogen (PN) were obtained by subtracting from the RN the corresponding amounts of nucleic acid by 1.69.

B. TECHNIQUE OF SAMPLING HUMAN TISSUES

The work in this section was done with material supplied by Dr. J.C. White, Postgraduate Medical School, Hammersmith, London. The sampling technique, which he used, was designed to maintain a supply of material,

which could be stored for periods of a few weeks without any risk of deterioration. It has been described elsewhere (Davidson, Leslie & White, 1951 a,b), and only the part relevant to the biochemical work will be dealt with here.

(a) Bone marrow: Small samples of 0.2-0.3 ml. of mixed marrow and blood were aspirated from the sternum of healthy volunteers and from patients suffering from various haematological disorders. The sample of known weight was evenly suspended in heparinized 0.85% sodium chloride solution, and, after removal of a small volume for the emumeration of nucleated cells in a Neubauer haemocytometer chamber, the remainder was used for microchemical determination of RNAP and DNAP. For storage purposes, this sample was immediately extracted first with ice-cold 10% TCA, as described above, and then with 80% ethanol. It was then suspended in 100% ethanol, in which it remained during transport and storage. The extraction of lipids was later completed, and the nucleic acid determinations carried out as described above for amounts of phosphorus below 40 ug.

(b) Peripheral blood: 2.5 ml. of freshly withdrawn venous blood were used for nucleic acid determinations, and 0.1 ml. diluted for nucleated cell count. Such samples were difficult to work with because of the

great excess of protein. In a number of cases analyses were performed on isolated leucocytes, which could be readily separated from leukaemic blood by heparinizing and standing at room temperature for 1-1½ hours. The leucocyte suspension was then separated from the sedimented erythrocytes. Alternatively, the leucocytes were separated from erythrocytes and plasma with the aid of bovine fibrinogen (Minor & Burnett, 1948).

Blood samples rich in reticulocytes were examined from the cases of haemolytic anaemia. It was found that all the reticular nucleoprotein was retained in the stromal precipitase, separated at pH 5.6 from the laked blood, and this material was analysed in the usual way.

Spermatozoa: Fresh seminal samples were obtained from a fertility clinic through the kind co-operation of Dr. Wachtel, of the Institute of Obstetrics and Gynaecology, University of London. Washing of the sperms with M/1000 sodium citrate in 0.85% sodium chloride and separation by centrifugation was carried out three times to remove seminal plasma; they were resuspended and an aliquot removed for dilution and sperm counting under the phase-contrast microscope, the remainder of the sample being used for nucleic acid assay. As before, the material was stored in 100% ethanol at 0°, following extraction with 10% TCA.

Other tissues: Through the kind co-operation of Dr. J.C. White and Dr. S. Sherlock, Postgraduate Medical School, Hammersmith, London, liver biopsy specimens from normal and pathological subjects were obtained and analysed for RNAP, DNAP and residual nitrogen (RN). Other surgical and pathological colleagues of Prof. J.N. Davidson kindly provided samples of human tissue obtained at operation or autopsy soon after death. These were used for isolation of nuclei by the citric acid method (Mirsky & Pollister, 1946), and an aliquot of the clean nuclei in suspension was removed for counting with a Neubauer haemocytometer. The remainder, and a sample of the whole homogenate, were extracted and analysed for LP, RNAP, DNAP and PN.

C. TECHNIQUE OF TISSUE CULTURE FOR BIOCHEMICAL INVESTIGATIONS.

Using the roller tube technique of Willmer (1942), Davidson & Waymouth (1943, 1945, 1946) had earlier demonstrated the conditions under which increases in total nucleic acid content could be obtained with chick heart explants from 10-12 day embryos. This technique was employed in its original form in studies on the RNAP and DNAP content of explants growing over periods of 10 to 192 hours. The results of this investigation were described in detail by Davidson, Leslie & Waymouth (1949) and only a few of the experiments will be reported here. For the subsequent work of correlating

changes in PN with those in ASP, LP, RNAP and DNAP, the tissue culture technique was considerably modified (cf. Davidson & Leslie, 1950 a,b, 1951), and will be described separately.

(a) Technique for tissue culture studies on RNAP and DNAP.

Fresh explants of 12-day chick embryo heart were grown in plasma clot in roller tubes. Each roller tube contained 24 pieces of tissue in three rows of eight embedded in clots of 0.1 or 0.05 ml. plasma per row according to the size of cultures employed. At first, relatively large pieces of tissue were used, and the total fresh weight of the 24 explants per tube was estimated at between 10-15 mg. It became apparent in the course of the work that true growth (i.e. a significant increase in cell number) could only be obtained with much smaller initial explants, and their size was reduced accordingly, bringing the total fresh weight of 24 down to about 2 mg.

Preliminary experiments had shown that in cultures maintained in Tyrode solution, the RNAP and DNAP fell to a fairly steady level, or 'resting state'. In all the subsequent tests, the explants were maintained for at least 20 hr. In Tyrode solution before the addition of growth-promoting medium. This 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 hrs.

Aseptic technique: In order to simplify the description of the preparation of tissue culture material, it should be mentioned at this stage that all the apparatus used was sterilized by autoclaving or by heating in a dry oven at 140° for 45 minutes; similarly, all the solutions were sterilized by autoclaving or by filtering through a Berkefeld candle, and their sterility was checked by the incubation at 37° of small samples in aerobic and anaerobic broth tubes. The tissue culture technique as a whole was carried out under aseptic conditions in a specially prepared room, in which every precaution was taken to eliminate air-borne dust and fluff.

Preparation of Tyrode solution: This physiological saline solution, which has been proved suitable for explanted chick tissues, was prepared periodically at double strength in volumes of 3-4 litres. In this way, it could be stored safely at 0°, and used as required for the preparation of normal strength solution.

Composition of double strength Tyrode solution in amounts per litre:

A.	NaCl	16.0 g.
	KCl	0.4 g.
	$CaCl_2$	0.4 g.
	$MgCl_2.6H_2O$	0.43 g.
	Dextrose	2.0 g.
B.	$NaHCO_3$	1.0 g.
	$NaH_2PO_4.2H_2O$	0.13 g.

The reagents in groups A and B were dissolved separately in boiled-out glass distilled water in the cold. The two solutions were mixed, and made up to the required volume with more glass distilled water. After adjusting, if necessary, to pH 7.8 by addition of N HCl dropwise, the solution was sterilized by filtering through a Berkefeld candle, the sterile filtrate being stored in a sterile Pyrex flask. The double strength Tyrode solution was transferred to Pyrex test-tubes in 20 ml. amounts, and was diluted (1:1) to normal strength with sterile glass distilled water.

Preparation of fowl plasma: The blood of a cockerel, previously fasted for 24 hours, was collected aseptically from the carotid artery in ice-cold waxed test-tubes. These tubes were stoppered and immediately centrifuged at 2000 r.p.m. for 20 minutes in a refrigerated centrifuge or in large buckets filled with crushed ice. The supernatant plasma was transferred by Pasteur pipette to ice-cold centrifuge tubes, tested for sterility, and stored for one or two days. The slight flocculent precipitate, which appeared in that time was removed by further centrifugation at 2500 r.p.m. for 30 mins. in the cold. The clear plasma was stored in waxed tubes at 0°.

Preparation of serum: Fowl serum was obtained by diluting 5 ml. of plasma with 10 ml. Tyrode solution,

and causing a clot to form by addition of a drop of purified bovine thrombin. By breaking up the clot, the serum could be drawn off with a pipette after centrifugation at 3000 r.p.m. for about one hour. Repeated extraction of the clot with Tyrode solution, gave 15-20 ml. of diluted serum for the original 5 ml. of plasma.

Preparation of embryo extract (EE): The embryo extract, used as growth-promoting medium, was prepared by aseptic manipulation from 12-day chick embryos by crushing them in ice-cold Tyrode solution with glass rods, removing the tissue residue by centrifugation, and freezing the extract hard to break up any cells in suspension. The extract, after thawing, was again cleared by centrifugation, and stored at 0° until required (cf. Davidson & Waymouth, 1943). The total N concentration of such extracts, when finally diluted with Tyrode solution, was 50-90 mg./100 ml.

Preparation of embryo extract-serum mixture (EES): In the preparation of the embryo extract-serum mixture (EES), 4 ml. of the original concentrated extract were diluted with 6 ml. of cockerel serum extract in Tyrode solution (final N concentration ca. 60 mg./ml.). Owing to the variations in plasma composition and in the concentration of EE and EES prepared in the course of different experiments, comparisons of results were only made under the fairly uniform conditions of one

particular experiment.

Test procedure: For each experiment 14 roller tubes were usually employed, 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. 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. With the larger explants, the results were expressed in terms of RNAP and DNAP content per tube or per 24 explants, while the contents per 2 tubes (or per 48 explants) were given in the case of experiments with smaller pieces of tissue.

In these tests it was not possible to evolve a statistical basis for determining the significance of results owing to variations in design and conditions from test to test. Instead reliance was placed on the proved reproducibility of a pattern of results, and the final increases, once corrected for plasma blanks, were considered significant only when they equalled or exceeded the corresponding increases in the plasma blank tubes.

(b) Technique for tissue culture studies on correlation between PN and phosphorus fractions during growth:

For this purpose considerable modification of

the original technique was required, involving, particularly, an increase in the number of explants per roller tube from 24 to 48. At the same time, the plasma clot in which cultures are generally grown, was largely eliminated by using a very thin film of plasma just sufficient to fix the explants to the wall. As the amounts of both phosphorus and nitrogen in this film were extremely small in comparison with the material in the 48 explants, it was possible to compare changes in the protein content of the explants with changes in the amounts of the various phosphorus constituents.

As a certain amount of contamination (by precipitation of protein etc. from the EES) was unavoidable, the 'inert' surface of the roller tube became coated with materials which caused increases in plasma blank determinations. With large numbers of explants growing in each tube, the increase in their area ensured that the whole wall was covered with living cells in a matter of four or five days; in such conditions the 'inert' area was reduced to a minimum. However, plasma tubes containing no explants were used in most of the experiments, and served to give warning of any abnormal contamination from embryo extract. As an additional means of reducing contamination to a minimum in some of the experiments, both the tubes containing the explants and those containing

plasma alone were incubated at 37° in Tyrode-serum mixture for 24 hours after the growth period. This removed part of the contaminating material from the tubes; it also ensured that the explants were taken for chemical analysis from the same medium (Tyrode-serum) as the original explants at zero time.

In the most recent experiments the plasma blank tubes were eliminated altogether. Instead, 30 to 40 tubes each containing 48 explants were set up in two consecutive days, and arranged in groups of 6-10 to allow a comparison of normal growth changes with those of explants growing under the influence of insulin and other hormones. With this standardised technique it was found possible to combine results from different tests, and so obtain groups of test and control results large enough to be analysed statistically.

The following gives in detail the various modifications which have been made to the original technique.

Roller tubes: Each experiment involved the use of 20 roller tubes each containing 48 fresh explants and, with a few exceptions, another 20 tubes containing only the plasma film clotted by a drop of sterile thrombin solution. The plasma film was formed by spreading about 0.05 ml. of fowl plasma over the

wall of the roller tube with the aid of a platinum wire. The stoppered tubes then stood vertically for at least four hours to allow the excess plasma to drain to the bottom for removal with a capillary pipette. The tubes were stoppered and kept in the cold until required (usually overnight).

Explants. Fresh explants were obtained as before from 12 day chick-embryo hearts. Throughout the whole process of cutting the hearts into the small pieces required for planting, the tissue was immersed in a tyrode-serum mixture (2:1). Forty-eight pieces were planted rapidly in 8 rows of 6 each, and the rubber stopper replaced immediately on the roller tube to prevent loss of moisture. Even so the tissue explants deteriorated unless the tubes were rotted with 1 ml. Tyrode-serum at 37-38° within 15 to 20 minutes of planting. This entailed leaving the explants just long enough in contact with the plasma film to allow them to adhere to the wall before the addition of the Tyrode-serum. At this stage each roller tube of 48 explants contained about 15 mg. fresh weight of heart tissue, as calculated on the basis of its total DNAP content (normally about 19 µg. DNAP/100 mg. fresh wt. of 12-day chick embryo heart). These explants usually remained for 24 hours in the Tyrode-serum at 37° before growth-promoting medium was substituted as fluid phase.

Growth-promoting medium. In past work the embryo extract was prepared under ice-cold conditions, according to generally accepted practice. However, much better results, in terms of more vigorous growth and reduced contamination, were obtained with embryo extract prepared by incubating the crushed chick embryos in Tyrode solution at 37° for 24 hours. (This procedure was adopted as a result of the demonstration by Sanford, Earle and Likely (1948) of the beneficial effect on culture growth from single cells obtained by the use of embryo extract, previously 'conditioned' by contact with growing cultures for 24 hours.) After centrifuging the pulped embryos for 20 minutes at 3000 r.p.m., the supernatant fluid was collected and yielded about 7 ml. of concentrated extract from each embryo. This was usually diluted with Tyrode solution to 10 ml., and the final test extract produced by adding a further 5 ml. of Tyrode-serum (2:1), giving a total N concentration of between 70 and 100 mg. per 100 ml. The incubated extract alone without added serum seemed to serve equally well as a growth-promoting medium, but it was the practice to use the incubated extract-serum (IES) to give the cultures every chance of remaining in a healthy condition throughout growth.

Test procedure: The material present in the 48 explants after they had been incubated for 24 hours at 37° served

as a base line for measuring the subsequent increases during growth. Throughout the tests the 1 ml. of growth-promoting agent (IES) in each roller tube was replaced by a fresh lot every 24 hours. Growth, as determined by DNAP increases (vide infra), was somewhat less extensive if the IES was renewed at 48 hourly intervals. The tubes were prepared for chemical analysis by removing the fluid and rapidly washing out each tube with physiological saline solution. In the case of explants sampled at the time of planting or in the early stages of growth, it was necessary to combine the contents of two or more roller tubes to ensure that there was at least 1.5 µg. DNAP in the alkaline digest used in the separation of RNAP and DNAP. With smaller amounts of DNA there was a danger of incomplete separation. At later stages it was possible to carry out the complete chemical analysis on the contents of a single roller tube, and to base results on separate duplicate determinations. The corresponding plasma blank tubes were treated in exactly the same way as the culture tubes.

D. DETERMINATION OF TISSUE GROWTH

Many of the difficulties associated with the concept of 'growth' are created by the different interpretations which can be placed upon the term.

It will help to simplify the situation, if the present discussion is confined to the problem of growth in explanted tissues. Even in this instance, it is a fairly complex process, and acceptable standards of growth and reliable methods for their measurement are essential if full advantage is to be gained from the tissue culture technique of growing cells under controlled conditions, in isolation from the animal body. (cf. Richards & Kavanagh, 1945; Waymouth, 1947).

We can look at the problem in two ways. Growth can be regarded in its biological aspect, and described in terms of changes in cell number and composition. Or, it can be judged by its general external manifestations, such as increase in area, volume or weight of the tissue explants. The essential difference is this; cell number and cell composition are elements inherent in the character of growth itself, the other aspects are primarily convenient dimensions chosen by the observer.

Again, tissue growth is not only a simple process of cell multiplication or cell enlargement, or a combination of these factors. From what is known of the dynamic nature of cell metabolism, as originally established by Schoenheimer and his colleagues (1942), it appears that the synthesis of much cell substance is inseparable from an accompanying process of

degradation. Even in growing tissues, the formation of new protein probably occurs as a result of the predominant character of the synthetic process rather than of decreased (or suppressed) protein decomposition (Friedberg, Schulman and Greenberg, 1948). When growth in tissue explants is considered, there is the additional complication that the biosynthesis of cells at one point is accompanied by a breakdown of cells at another, and growth is thus represented by the new tissue synthesized in excess of tissue lost by cell destruction.

There are serious limitations in the measurement of such growth in terms of its external manifestations. While these can give some indication of the general effect of growth-promoting medium and chemical agents on cellular proliferation, the internal changes, such as an alteration in cell size, remain hidden and are likely sources of error. In addition, migration of cells must often affect area measurements in an unpredictable way. (cf. Willmer, 1933),

It would seem more reasonable to base measurements on the increase in some metabolic aspect of the cultures, as, for example, oxygen consumption or lactic acid production (Lipmann & Fischer, 1932), phosphorus uptake (Wilson, Jackson & Brues, 1942; Brues, Rathbun & Cohn, 1944), or nucleoprotein

phosphorus (NPP) formation (Willmer, 1942; Davidson & Waymouth, 1943, 1944, 1945, 1946). The last method proved most promising, and on the publication by Schmidt & Thannhauser (1945) of their method for determining the amounts of RNAP and DNAP in tissues, it became possible to follow the variations in amount of the two nucleic acids during the growth of chick heart explants (Davidson, Leslie & Waymouth, 1949).

Nearly all the methods discussed so far deal with one single feature of growth, which is taken to represent the process as a whole. But there are three important and distinct aspects of tissue growth; these are

(1) change in cell number,

(2) change in amount of cell substance, and

(3) change in amount of intercellular substance.

During growth, for example, cell number may increase, but cell size remain unchanged or even diminish. Alternatively, there may be growth in cell size without cell division, the two process showing at least some degree of independence (Willmer, 1935).

With these complexities in mind, Cunningham & Kirk (1942) proposed that culture growth should be measured by three parameters; the increase in cell number, the average change in cell size, and the volumes in which the cells exist at various

stages of growth. As a direct outcome of the work reported in this section, a chemical counterpart of this method has been developed, and is applicable to both tissue culture and embryological studies.

The method is founded on the belief that growth can only be adequately described by measuring simultaneously the changes in cell number and in average cell composition and mass (Davidson & Leslie, 1950 a,b, 1951 a,b). These determinations depend on the high degree of constancy in the amount of DNA in the somatic cells of one animal. As a result, the total amount of DNA in the tissue is directly proportional to the cell number, which can be calculated if the absolute amount of DNA per nucleus is known; and once the ratio of tissue mass or chemical content to DNA is known, it is an easy matter to calculate the average mass and average composition of the cells. This method, of course, does not lessen the importance of microscopical studies on changing cell structure and character, for it can be very usefully combined with such studies to the benefit of our understanding of cellular growth.

There are certain reservations, of course, regarding the use of this method. When the initial explants are small, and growth relatively extensive,

it is likely that an appreciable proportion of the cells will be approaching or undergoing mitosis. While this is an advantage in studying the changes in cell composition involved in the process of cell division, it is likely that the average DNA content of the nucleus is raised to some extent, as is apparently the case in rapidly proliferating marrow cells. (Section V). It has not been possible to determine the extent of this increase in the explanted cells, and the assumption has been made that the average amount found for adult and embryonic chick cells (2.35 µg. x 10^{-7} DNAP/nucleus) is a close approximation to the average in the cells of the chick heart explants.

In rapidly growing tissue, however, it will mean that estimates of cell number based on DNAP content will be slightly higher than the actual number of individual cells present in the tissue. There is likely to be less effect on the results of cell composition, since the ratios to DNA must always indicate the changes in cell composition which occur in the sequence of events leading to cell division.

D. TECHNIQUE FOR STUDYING BRAIN, HEART, LIVER, AND MUSCLE IN THE DEVELOPING CHICK EMBRYO.

Eggs from a stock of pure bred Leghorn poultry were received in batches weekly, after seven

days incubation and were further incubated at 37° to 38° in a Hearson egg incubator. Embryos were taken for chemical analysis from the eighth day of incubation, until two days following hatching. The common methods of determining embryonic age from the weight of the embryo or from the period of incubation are both subject to errors, but it was decided to adopt the second method and to base results for ten to twenty day embryos on the means of groups of determinations made on successive days. For example, the figures for 19.5 day embryos are the means calculated for embryos sampled on the 19th and 20th days of incubation. The investigation was carried on at irregular intervals over a period of two years and the results cover any seasonal variations which may occur in embryonic development.

Immediately after removal of the embryo from the egg, the brain, heart, liver and a portion of skeletal muscle from the leg were removed, chilled in ice and weighed. In the youngest embryos (8 days) the determinations were made on pooled material from twelve embryos; two to six embryos of 10 to 18 days provided sufficient material for analysis, and after 18 days the determinations could be carried out on organs from individual embryos. Prior to extraction with 10% TCA, the weighed tissues were thoroughly crushed or homogenized in a Potter-Elvehjem homo-

genizer, and transferred quantitatively to centrifuge tubes for the determination of ASP, LP, RNAP, DNAP, PN and non-protein nitrogen (NPN) by the procedure described above.

SECTION III.

BIOCHEMICAL STUDIES ON CHICK HEART EXPLANTS GROWING IN VITRO

A. INTRODUCTION

There are three distinct stages in the development of the work to be described below. In the first it was established that the initial explants had to be sufficiently small to ensure that the increase in tissue by cell proliferation was far in excess of tissue lost by cell destruction at the centre of the explants. There followed the work to develop a technique for measuring protein nitrogen in addition to the phosphorus fractions in the explants and, in the course of this, a standardised procedure was established for studying the changes in cell number and composition during growth periods of 6 or 7 days. The final section shows how the procedure has been applied in an investigation on the influence of insulin on the growth of explanted tissue.

B. RESULTS

1. CHANGES IN RNAP AND DNAP CONTENT OF CHICK HEART EXPLANTS

The work in this section was carried out with the roller tube technique used by Davidson & Waymouth in their earlier studies (1943, 1944, 1945, and 1946). As described above, results are expressed in amounts of RNAP and DNAP per 24 explants, that being the number growing in plasma clots in each roller tube. All the amounts stated have previously been corrected for the RNAP and DNAP found in the plasma blank tubes at corresponding stages of growth.

(a) Changes occurring in explants after planting:

Earlier work (Davidson & Waymouth, 1943, 1945) had shown that fresh chick-heart cultures lost an appreciable amount of their total nucleic acid phosphorus (NPP) when they were maintained in Tyrode solution for 2 or 3 days after planting.

When the RNAP and DNAP content of the cultures were measured, the ratio RNAP/DNAP was found to lie between 2.2 and 2.8 at the time of planting. 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. By the

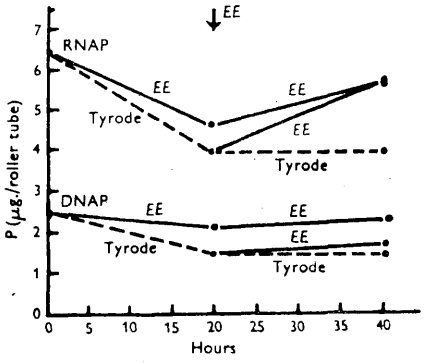


Fig. 1. Changes in ribonucleic acid P (RNAP) and deoxyribonucleic acid P (DNAP) of chick-heart explants *in vitro*, using either Tyrode solution or embryo extract (EE) as the fluid phase. The figures for P represent the amounts found in the two nucleic acids in 24 pieces of tissue (planted in three rows 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.

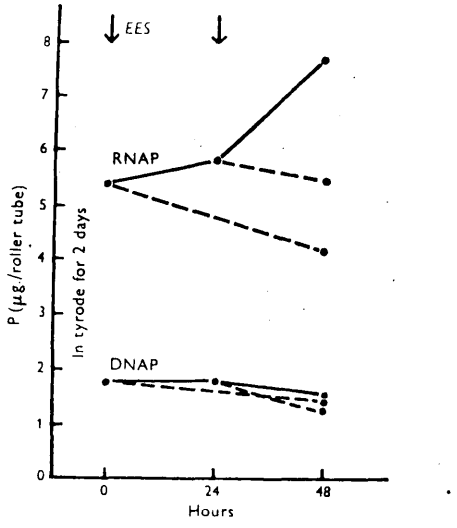


Fig. **2.** Effect of colchicine (1 in 4,000,000) on the changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro*, using EES as the fluid phase. The broken lines show the changes occurring when colchicine is present in the EES. After maintaining the cultures in Tyrode solution for 36 hr., EES or EES containing colchicine is added to the tubes at the times indicated by the arrows.

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, but to a smaller degree, if embryo extract was used instead of Tyrode solution in the period immediately following planting. (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. As a result of the experience gained in such experiments, the practice of keeping freshly planted cultures in Tyrode solution for 20-24 hours before adding the growth-promoting medium was adopted in all the experiments described below.

(b) Inhibition of growth of explants 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 a means of confirming that increases in RNAP and DNAP in

the presence of growth-promoting medium were indeed due to synthetic processes associated with the growth of new tissue.

The results of a typical experiment with colchicine are seen in Fig. 2. 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 similarily diluted with Tyrode solution.

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 the 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.

(c) Changes in RNAP and DNAP contents of relatively large explants:

In experiments in which relatively large explants were in contact with growth-promoting medium for periods up to 48 hours, there was a conspicuous failure to obtain a rise in DNAP, commensurate with the rise in RNAP or the increase in area of the cultures. It was first supposed that the increase in DNAP might be delayed, and changes in RNAP and DNAP were accordingly followed in cultures growing for periods of 6-7 days.

In the course of these tests the embryo extract-serum mixture (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 tended to become coated with precipitate, and the blanks to become unduly large.

Fig. 3 shows the typical results obtained in a test of this nature, and in Plate 1 are photomicrographs showing visible changes in the cultures. At the end of 120 hr., the cultures had spread to

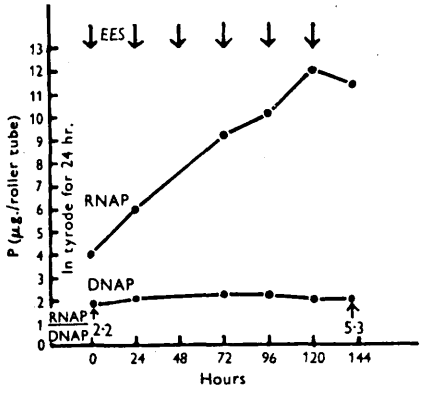


Fig. 3 Changes in RNAP and DNAP in relatively large chick-heart explants growing *in vitro* over 144 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows).

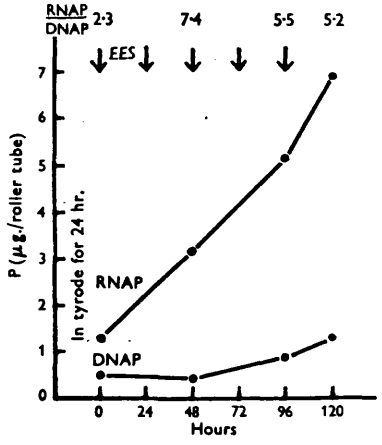


Fig. 4. Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 120 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows). Results based on 48 pieces of tissue from two roller tubes

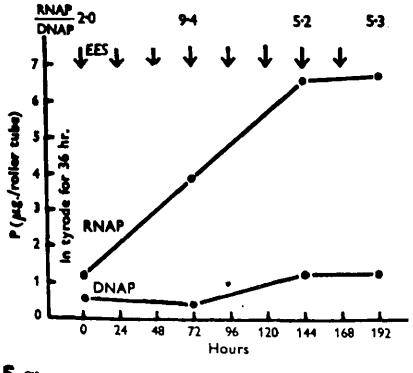


Fig. 5. Changes in RNAP and DNAP in very small chick-heart explants growing *in vitro* over 192 hr. following addition and renewal of EES at intervals of 24 hr. (as shown by arrows). Results on the same basis as Fig. **4.**

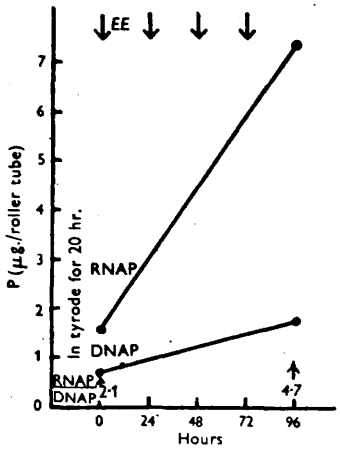


Fig. **6.** Effect of replacing EES by embryo extract alone in maintaining growth of very small chick-heart explants *in vitro*. The changes in RNAP and DNAP are followed over 96 hr., the EE being renewed every 24 hr. (as shown by arrows). Results on the same basis as Fig. **4.**

PLATE I.

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.

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 core of the explants was most noticeable, the DNAP content fell. This behaviour was again reflected in the RNAP/DNAP ratio, which increased from 2.2 to 5.3.

In tests of this type, 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 was established that the rise in RNAP was usually of the order of 250-300%, while the DNAP only increased in the early stages, at the most 25% above the initial resting value. This rise in DNAP was temporary and was reduced after 96 hr. in spite of the continued high RNAP content at this stage.

(d) Changes in RNAP and DNAP content of small initial explants:

In view of the failure to obtain an increase

in the DNAP content of the explants even in tests of long duration, a new approach was tried. 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. This reduction could be explained on the assumption that any increase in DNAP by the production of new cells was exceeded by its loss as a result of necrosis in 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 the roller tubes in these tests) was insufficient to provide for the continued existence of the relatively large cultures employed.

Support for these interpretations was available in the observations of Brues, Rathbun & Cohn (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 found that in such a medium, 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.

The technique was accordingly modified with the object of reducing the initial size and weight of the tissue cultures to a minimum (as described in Section II). 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 were based on the mean of two determinations, and as shown in Tables 9 and 10 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 varied only slightly between 0.65-0.75 and 0.25-0.35 µg. P per roller tube, respectively. These could not easily be reduced further since a lower limit is imposed

Table 9. *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 10. *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

† Plasma/2 tubes.

on the size of the cutting and planting technique. With some practice it was possible to cut the tissue into pieces which had 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. of fresh EES every 24 hr.

With this modified technique substantial increases in the amounts of both RNAP and DNAP were obtained. In Fig. 4 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 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 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.

Tests were run for longer periods in order to find the maximal values to which the RNAP and DNAP could rise under these experimental conditions. The results are shown in Fig. 5. Both the 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. 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 was obtained in all the experiments in which substantial increases in both RNAP and DNAP were obtained with this technique. The other significant observation, which is a feature both of these and the later tests with the improved technique, is the delay in the rise of DNAP for 48 or 72 hours, even though the RNAP has increased appreciably in the same period.

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

Most of the tests so far described have been carried out with embryo extract and serum mixture (EES) as the growth-promoting medium. However, other tests showed clearly that EE alone was fully adequate for the purpose, as can be judged from the results of one such test given in Fig. 6. 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. Good growth occurred over a period of 96 hr., and was 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. 4). It is concluded that EE alone as fluid phase is sufficient to supply all the necessary materials for growth of fibroblasts in vitro.

2. CHANGES IN PROTEIN NITROGEN (PN) AND PHOSPHORUS FRACTIONS OF CHICK HEART EXPLANTS:

The roller tube technique was modified, as described in Section II, to enable determinations to be made of PN as well as the phosphorus fractions, acid-soluble phosphorus (ASP), lipid phosphorus (LP), RNAP and DNAP. This involved the increase in the number of explants per roller tube from 24 to 48, the virtual elimination of the plasma clot by using only a very thin film of plasma to fix the explants to the wall, and an improvement in the character of the growth-promoting medium. In the early stages of the experiments determinations were carried out on the combined contents of two or more test or plasma blank tubes, but as the amounts of tissue increased, the contents of one roller tube were generally sufficient. All

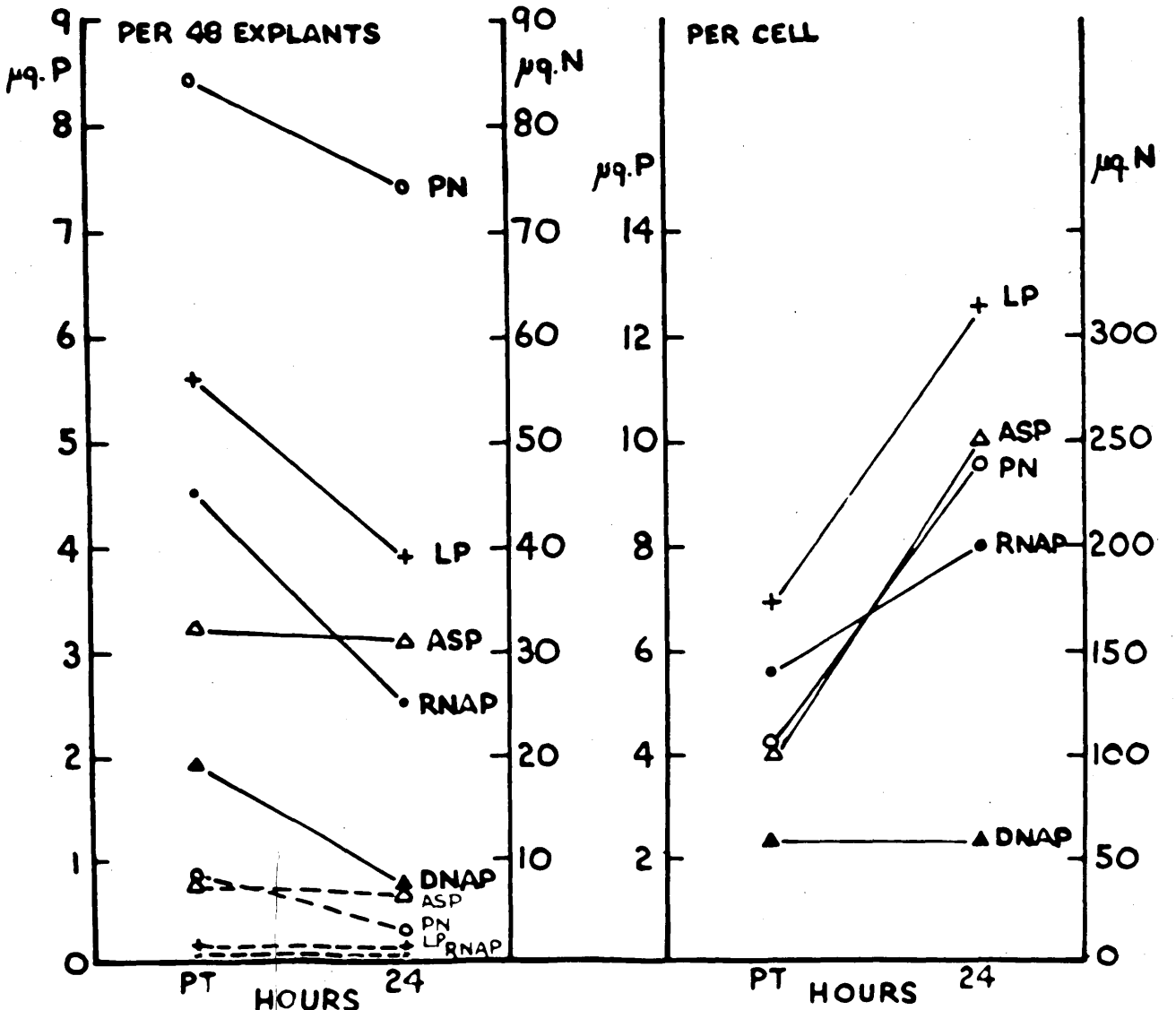
results are expressed as the amounts of phosphorus or nitrogen per 48 explants and per cell.

(a) Changes in cell number and composition of explants after planting:

Fig.7 shows what happened in a series of tissue culture tests when the explants were incubated with Tyrode-serum for 24 hours after planting. The left-hand section of the diagram gives the actual amounts of the various constituents present in 48 explants at the time of planting (PT) and after 24 hours in Tyrode-serum. These results have been corrected for plasma blanks, which are shown as dotted lines below; no DNAP was found in the plasma blanks in any of the tests. Under these conditions the main constituents of the explants, with the exception of the ASP, are greatly reduced in amount. The ASP and PN are distinguished by the relatively high amounts found in the plasma blanks as compared with the negligible amounts of the other constituents. The ASP in the blank amounts to 25% of that present in the explants, and the protein remains less than 10% of the explant protein.

From the decrease in the amount of DNAP per 48 explants it can be deduced that the number of cells has diminished to about one-third of their original number. When results are expressed as amounts per cell

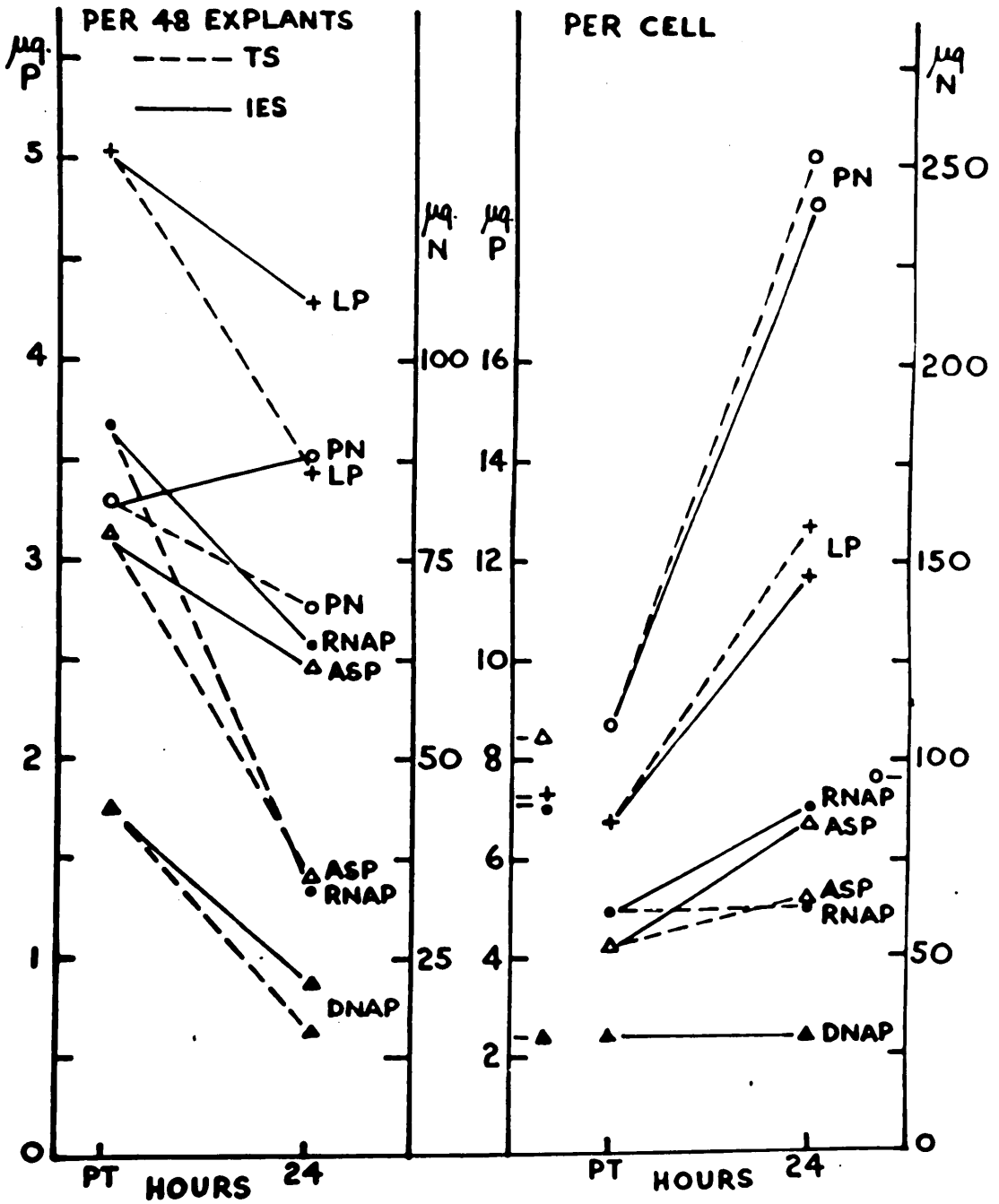
Fig. 7. Chemical changes in chick heart explants during 24 hours in TS immediately following planting in roller tubes. Left section - amounts of constituents per 48 explants (after correction) or per roller tube in case of plasma blanks. Results for 48 explants - unbroken lines; plasma blank results - broken lines. Right section shows content of various constituents per cell based on DNAP content per cell of 2.35×10^{-7} ug.
PT - planting time.


PER 48 EXPLANTS
µg. P
µg. N
PN
LP
ASP
RNAP
DNAP
PT
HOURS
24
PER CELL
µg. P
µg. N
LP
ASP
PN
RNAP
DNAP
PT
HOURS
24

(taking the DNAP content of the chick cell nucleus as 2.35×10^{-7} µg.), they reveal the relative changes in tissue composition which accompany these losses in material. These are illustrated on the right hand section of Fig. 7. Three of the constituents, ASP, LP and PN increase their content per unit of DNAP by 100% or more; the RNAP in contrast rises only 30%. Examination under the microscope at this stage (i.e. after 24 hours in Tyrode-serum) usually revealed a narrow band of migrating cells round the explants.

In one set of explant tubes a comparison was made of the effects of adding TS and IES to the explants immediately after planting (Fig. 8). The left-hand section of the diagram shows that the losses in material were much greater in the case of Tyrode-serum (TS), and that the IES by reason of its growth-promoting activity was able to diminish, but not prevent, these losses. When the changing composition of these explants is examined (right-hand section), it is clear that the type of fluid medium has had little effect on the amounts of LP and PN per unit of DNAP, for at the end of 24 hours they are increased to the same extent by both treatments. The presence of growth-promoting medium has, however, increased the amounts of RNAP and ASP per cell, which in this test were little changed during the 24 hours

Fig. 8. Comparison of chemical changes in explants when in contact with either TS or IES during the 24 hours immediately following planting in roller tubes. Left section - amounts of constituents per 48 explants after correction for plasma blanks. Right section - content per cell for the same constituents (as in Fig.7). Unbroken lines - explants in contact with IES; broken lines - explants in TS. PT - planting time.


PER 48 EXPLANTS
μg P
TS
IES
5
4
3
2
1
0
LP
PN
LP
PN
RNAP
ASP
ASP
RNAP
DNAP
μg N
100
75
50
25
μg P
16
14
12
10
8
6
4
2
PT
HOURS
24
PER CELL
μg N
250
200
150
100
50
0
PN
LP
RNAP
ASP
ASP
RNAP
DNAP
PT
HOURS
24

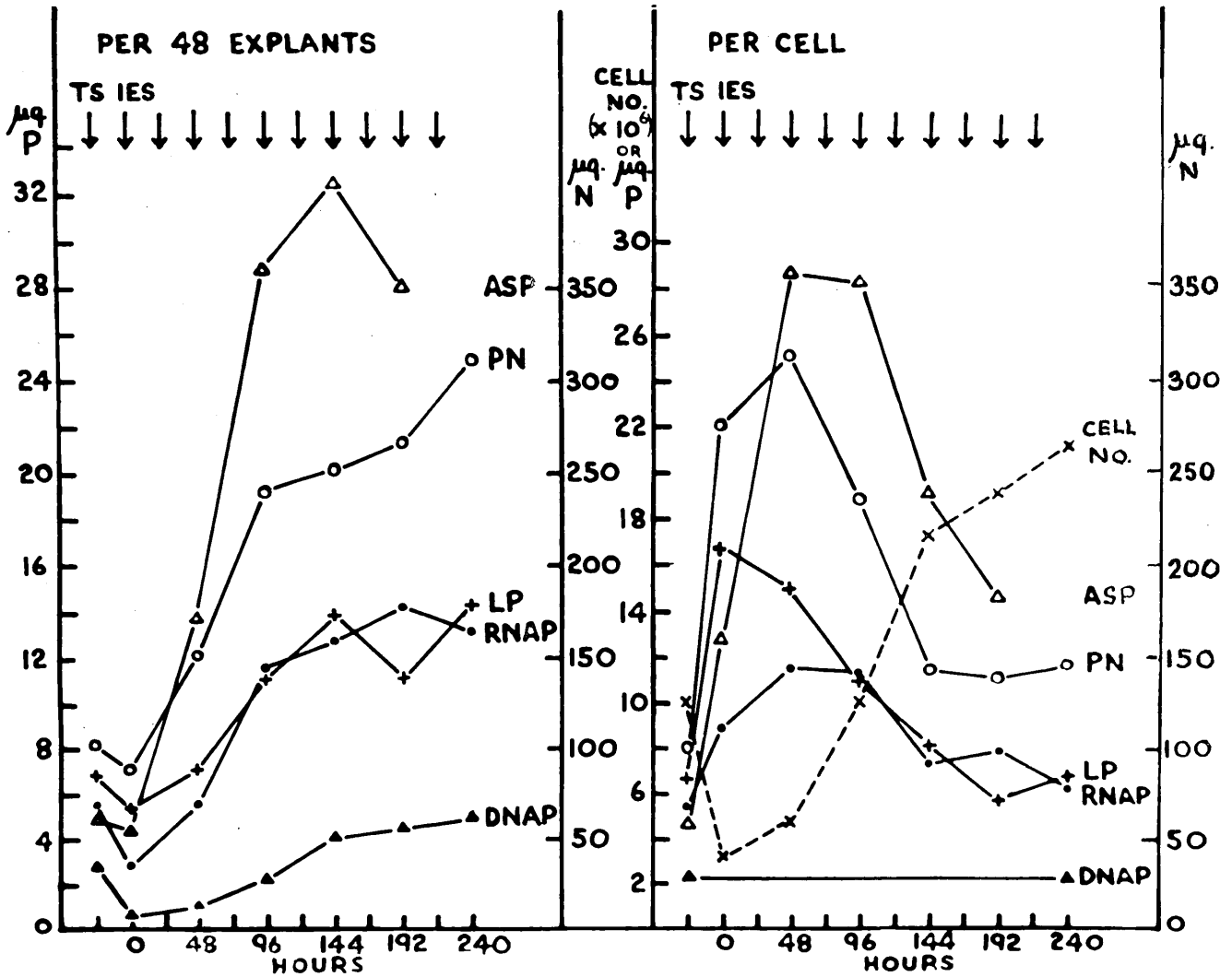
in Tyrode-serum.

From these results it is evident that a striking change in explant composition occurs in the 24 hours following planting. In relation to the constant DNAP, the PN and LP contents are more than doubled, whatever the medium. Because of the relative quantities involved this cannot be the result of retention of material from the plasma or of contamination from the fluid medium; it is concluded that these increases are part of the activity of the explants and that the additional material in the cells comes from damaged cells or from the fluid phase. The RNAP and ASP also appear to be closely associated fractions. In TS they will increase per cell or remain unchanged throughout 24 hours, while the addition of growth-promoting medium (IES) increases their amounts per cell over those found in the case of TS.

(b) Changes during growth of the explants:

In all experiments on the effect of growth-promoting agent on cell number and cell composition of these explants, it has been the practice to keep the cultures in TS for the first 24 hours in order to establish a base-line for subsequent measurements. Fig. 9 contains the results of a single test, which is typical of many that have been carried out. In the left hand section the dotted lines between the points at

Fig. 9. Chemical changes in chick heart explants grown *in vitro* during Test G.17. Left section - amounts per roller tube of 48 explants without correction for plasma blanks. Right section - amounts per cell without correction for plasma blanks (based on DNAP as for Fig.1). Unbroken lines show amounts of constituents; broken line shows cell number. Hours indicate time during which explants were in contact with TS or IES; they were treated with TS for 24 hours after each period of growth in IES.


PER 48 EXPLANTS
TS IES
µg P
32
28
24
20
16
12
8
4
0
ASP
PN
LP
RNAP
DNAP
0
48
96
144
192
240
HOURS
CELL NO. (x 10⁶) OR µg. N µg P
30
26
22
18
14
10
6
2
350
300
250
200
150
100
50
PER CELL
TS IES
µg. N
CELL NO.
ASP
PN
LP
RNAP
DNAP
0
48
96
144
192
240
HOURS

planting time (PT) and zero time(ZT) show how the amounts of the various fractions per 48 cultures have altered in Tyrode-serum, and how their absolute values are very much smaller than those obtained at later stages of the test. In this graph no correction has been made for plasma blanks, and the results represent the actual amounts present per roller tube of 48 explants. Every 24 hours throughout growth (as indicated by the arrows) the IES was replaced by fresh 1 ml. lots. When tubes were taken for chemical analysis in this series of experiments, they were incubated after their growth period for an additional 24 hours in contact with 1 ml. of Tyrode-serum. Preliminary experiments had shown that this produced only small changes in the number or composition of the culture cells, and that it helped to reduce the plasma blanks.

The increase in DNAP is most pronounced between 48 and 144 hours of growth, and is succeeded by a slower rise during the last four days of the test. By 144 hours the culture cells had covered the entire surface of the roller tube, and further expansion could only arise by increases in the number of layers or in the density of the cells. From microscopic examination it appeared that many layers of cells had formed, and that cells quite different in appearance from the usual spindle-shaped fibroblasts became increasingly prominent

during the last four days of growth. A photomicrograph of a section of these cultures (Plate 2) shows the distinction between the two types, one, spindle-shaped, is the normal fibroblast, the others, irregular in shape and sometimes vacuolated, have the characteristics of macrophages. None of these was seen in plasma blank tubes (as confirmed by absence of any significant increase in DNAP in Table 11), and they could not have come from the embryo extract. These results confirm the earlier observations of Ephrussi and Hugues, (1930) and Latta and Bucholz (1929) that the transformation of fibroblasts to macrophages will occur during growth in vitro.

While the DNAP (and, therefore, cell number) continued to increase rapidly until 144 hours, the greatest accumulation of PN, ASP, RNAP and LP took place in the first 96 hours. Any further increases in these constituents were small by comparison, and in the case of PN may have been caused by contamination from the IES, which produced in the plasma blanks PN values at 144 to 240 hours representing between 14 and 30% of the amounts found in the tissue culture (Table 11).

The results for the same test after correction has been made for the plasma blanks are given on the left of Fig. 10. The amounts of DNAP, RNAP and LP remain much the same as before correction, and the

PLATE 2.

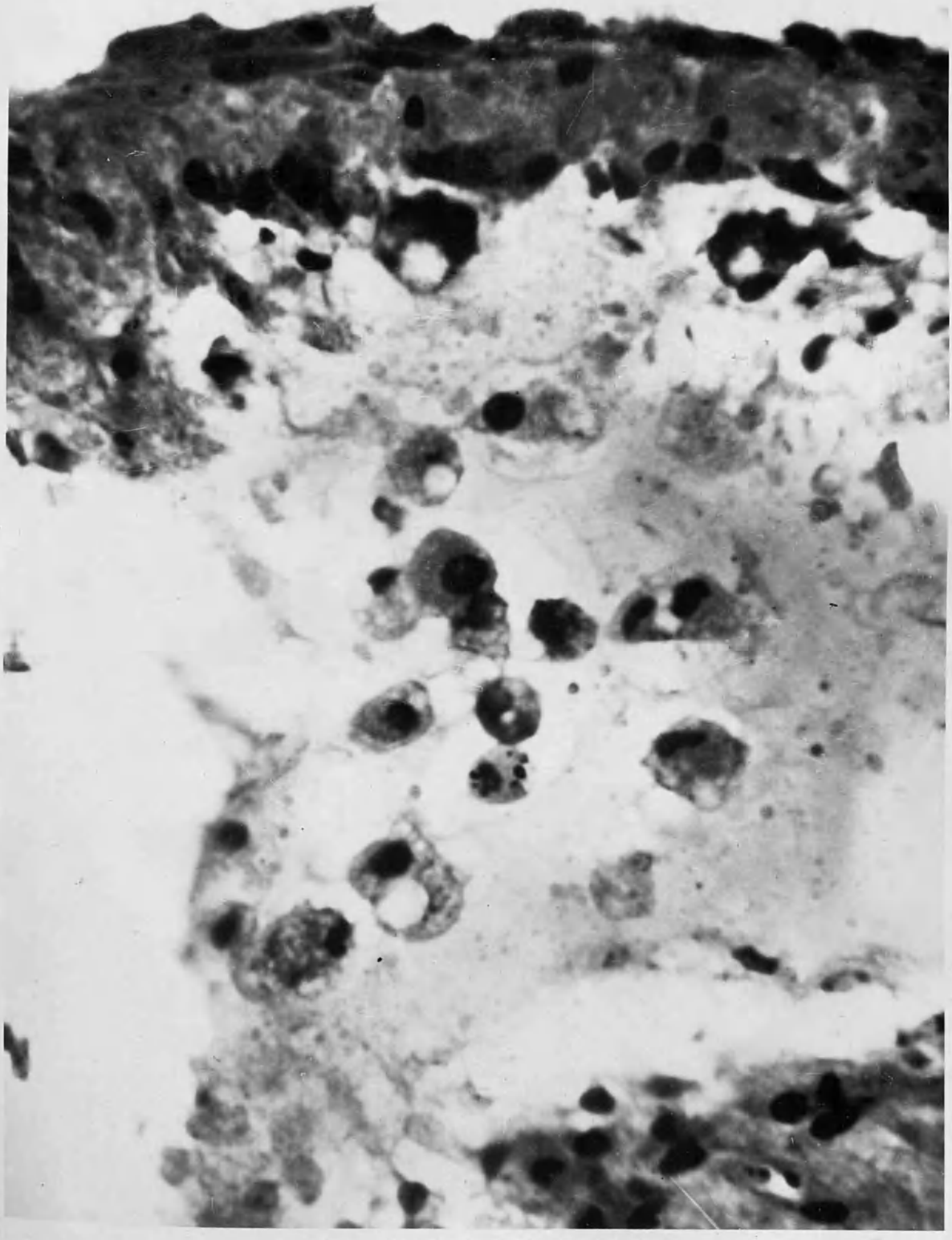


Table 11.

Results of analyses of contents of roller tubes in Test G 17. Figures for plasma blanks are actual amounts found per roller tube. Those for 48 explants have been corrected for amounts found in corresponding plasma blank tubes. In each case results are expressed as µg. P or µg. N. The time is given in hours during which explants or plasma films were in contact with TS or IES:

Roller Tube	Fluid phase	Time hours	µg. P or N per roller tube						
			ASP	LP	RNAP	DNAP	NAP	P	PN
Plasma blank	-	-	2.29	0.14	0.07	0.00	0.00	2.5	7
Plasma "	TS	24	1.74	0.03	0.03	0.00	0.03	1.8	0
Plasma "	IES	48	4.31	0.05	0.16	0.00	0.16	4.5	13
Plasma "	IES	96	5.52	0.63	0.06	0.00	0.06	6.2	27
Plasma "	IES	144	9.72	0.67	0.46	0.00	0.46	10.9	52
Plasma "	IES	192	5.12	0.75	0.05	0.06	0.11	6.0	38
Plasma "	IES	240	-	0.94	0.16	0.00	0.16	-	98
48 Explants in plasma	-	-	2.50	6.66	5.44	2.38	7.82	16.98	94
48 Explants	TS	24	2.39	5.38	2.86	0.76	3.62	11.39	89
48 Explants	IES	48	9.43	6.95	5.36	1.13	6.49	18.55	138
48 Explants	IES	96	23.2	10.5	11.37	2.39	13.76	47.46	223
48 Explants	IES	144	25.6	13.2	12.4	4.1	16.5	55.3	200
48 Explants	IES	192	22.8	10.3	15.1	4.5	19.6	52.7	228
48 Explants	IES	240	-	13.4	13.1	5.0	18.1	-	215

main alterations are confined to reductions in the amounts of PN and ASP. In these circumstances the PN of 48 cultures appears to have reached its maximum at 96 hours, and to have remained at this level in spite of the more than twofold increase in cell number which followed during the next six days.

(c) Changes in cell number from time of planting:

Calculation of cell numbers on the basis of the constant DNA content of the nucleus shows that two-thirds of the cells present at the time of planting were lost in the first 24 hours in TS (Table 12). Between 48 and 144 hours the numbers approximately doubled in each 48 hour period, followed by the slow rise to the maximum of 21 million per 48 explants. There were consequently seven times as many cells per roller tube at 240 hours as were present at zero time.

(d) Changes in cell composition from time of planting:

The changes in cell composition which accompany these successive phases of culture growth, are shown on the right of Figs. 9 and 10 which respectively give the results before and after correction has been made for plasma blanks. In each figure the cell number, expressed as millions per 48 cultures, is drawn as an unbroken line between the time of planting

Table 12.

Cell number and protein nitrogen/phospholipid phosphorus (PN/LP) ratios during tissue culture growth. PN/LP ratios refer to following tests - 1. based on results of G 17 (Table 11); 2. based on pooled results (Fig.11).

The time is given in hours during which explants were in contact with TS or IES.

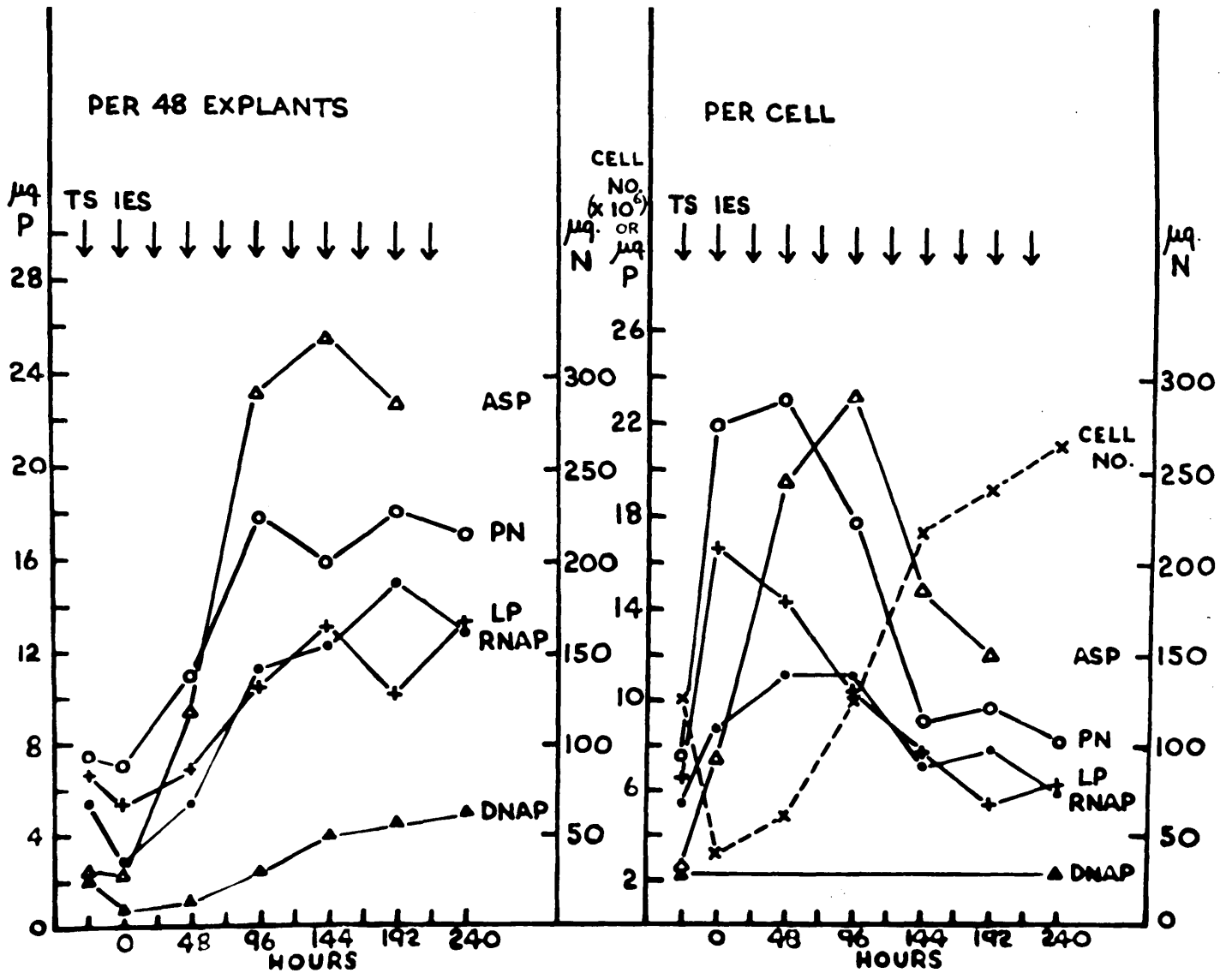
Explants	Medium	Time hours	Cell number (x 10^6)	Ratios PN/LP (1)	PN/LP (2)
48	-	-	10.1	13.7	14.1
48	TS	24	3.2	19.1	16.5
48	IES	48	4.8	18.1	19.9
48	IES	96	10.1	18.6	21.2
48	IES	144	17.3	18.5	15.1
48	IES	192	19.0	22.4	22.4
48	IES	240	21.2	16.1	16.1

and zero time, and as a broken line for the rest of the test. The other lines show in contrast the changes in the amounts per cell of ASP, LP, RNAP and PN, when the DNAP is taken as constant at 2.35 µg. per cell.

A comparison of the two figures (Figs. 9 and 10) makes it clear that the correction for the plasma blank makes no difference to the pattern of the changes in cell composition, although it does reduce the amounts of ASP and PN per cell considerably. In both cases the 24 hours in TS produced abrupt increases in PN and LP, which brought these constituents to near their maximal amounts per cell. After 48 hours both PN and LP were decreasing per cell until they reached a fairly constant level between 144 and 240 hours. On the other hand, ASP and RNAP showed more prolonged increases, only reaching their maximal amounts per cell between 48 and 96 hours. Later, the RNAP and, possibly, the ASP fell to a constant amount per cell in the last four days of growth.

The high values for ASP and RNAP per cell coincided with the phase of rapid multiplication of the cells. By the end of that phase the PN and LP of the cells had fallen to a final steady level, and the cells continued to multiply more slowly with little change in composition. Along the ordinates

Fig. 10. Chemical changes in chick heart explants grown *in vitro*. during Test C 17 after amounts per 48 explants have been corrected for corresponding plasma blanks. Otherwise, as for Fig. 9.


PER 48 EXPLANTS
μq P
TS IES
28
24
20
16
12
8
4
0
μq. N
300
250
200
150
100
50
ASP
PN
LP RNAP
DNAP
0
48
96
144
192
240
HOURS
CELL NO. (x 10^6) OR μq P
26
22
18
14
10
6
2
PER CELL
CELL NO.

are points showing the amounts of the various constituents of the cells as determined in the original heart tissue from which the explants were prepared. Apart from the ASP fraction, there was little difference between the composition of the explants (after correction for plasma blanks) at 240 hours and the composition of the embryonic heart tissue.

(e) Effect of initial size of explants on extent of growth:

In this culture technique one variable, which affects the extent of culture growth, is the initial size of the explants. In any one test the pieces will be of an average size which determines the amount of DNAP or number of cells per roller tube at zero time. It is, however, impossible to cut them to the same average size in every test, and the initial cell number varies from one test to another. Table 13 contains results for cell number at zero time and after 144 hours in contact with IES in five different tests. In all the cell number increased 6 or 7 times, the final numbers keeping in the same proportion to one another as those initially present. They illustrate that the growth of cultures under these conditions is proportional to the initial size of the explants, provided that they are small enough initially to prevent deterioration of

Table 13.

Comparison of initial and final cell numbers per 48 chick heart explants in a series of tissue culture tests.

Explants	Medium	Time hours	Cell Number ($x10^6$)					Ratios				
			1	2	3	4	5	1	2	3	4	5
48	TS	24	1.4	1.6	2.7	3.0	3.2	1	1	1	1	1
48	IES	144	7.6	11.5	13.6	14.5	17.3	5.4	7.2	5.1	4.6	5.4
48	IES	240	-	-	-	22.6	21.2	-	-	-	7.5	6.6

the cells at the centre of the explants.

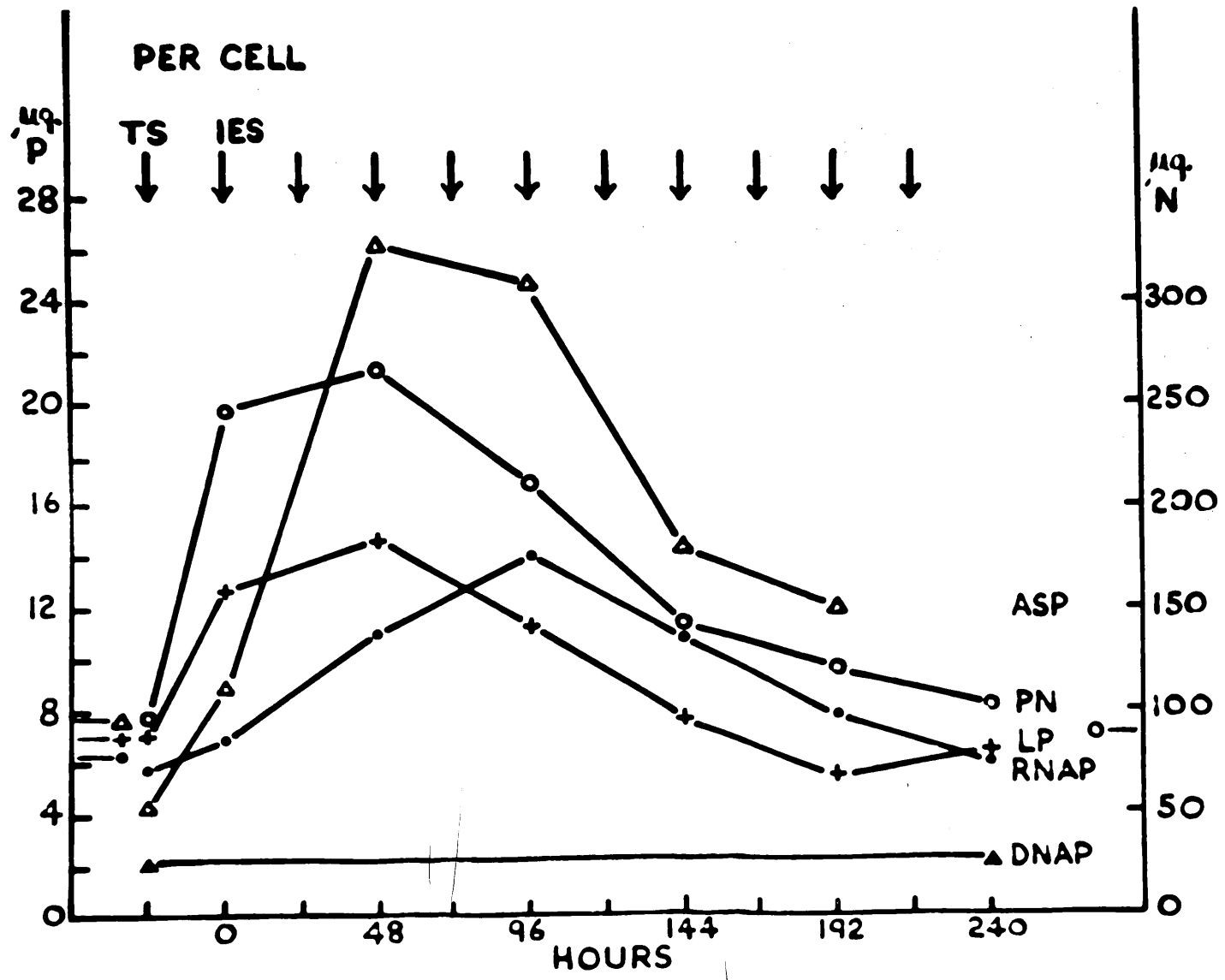
(f) Uniformity in pattern of changes in cell composition during growth:

Although the amounts of the materials per 48 cultures may vary from one test to another, results expressed in terms of amounts per cell are closely similar for corresponding periods of growth. As a result it is possible to combine the results from a series of tests to produce a more generalised account of the changes in cell composition of chick heart explants. This is given in Fig. 11. It largely reproduces the pattern shown for one of the tests, but emphasises the close association between PN and LP on the one hand, and between ASP and RNAP on the other. The rise and fall in content of PN and LP per cell coincided, the ratio between the two having kept fairly constant throughout growth (Table 12). As before, the ASP and RNAP attained their maximal amounts per cell over the period 48 to 96 hours.

(g) Composition of explants in contact with growth-promoting medium:

In the tests just described the explants have been kept for 24 hours in Tyrode-serum after their periods in contact with growth-promoting medium.

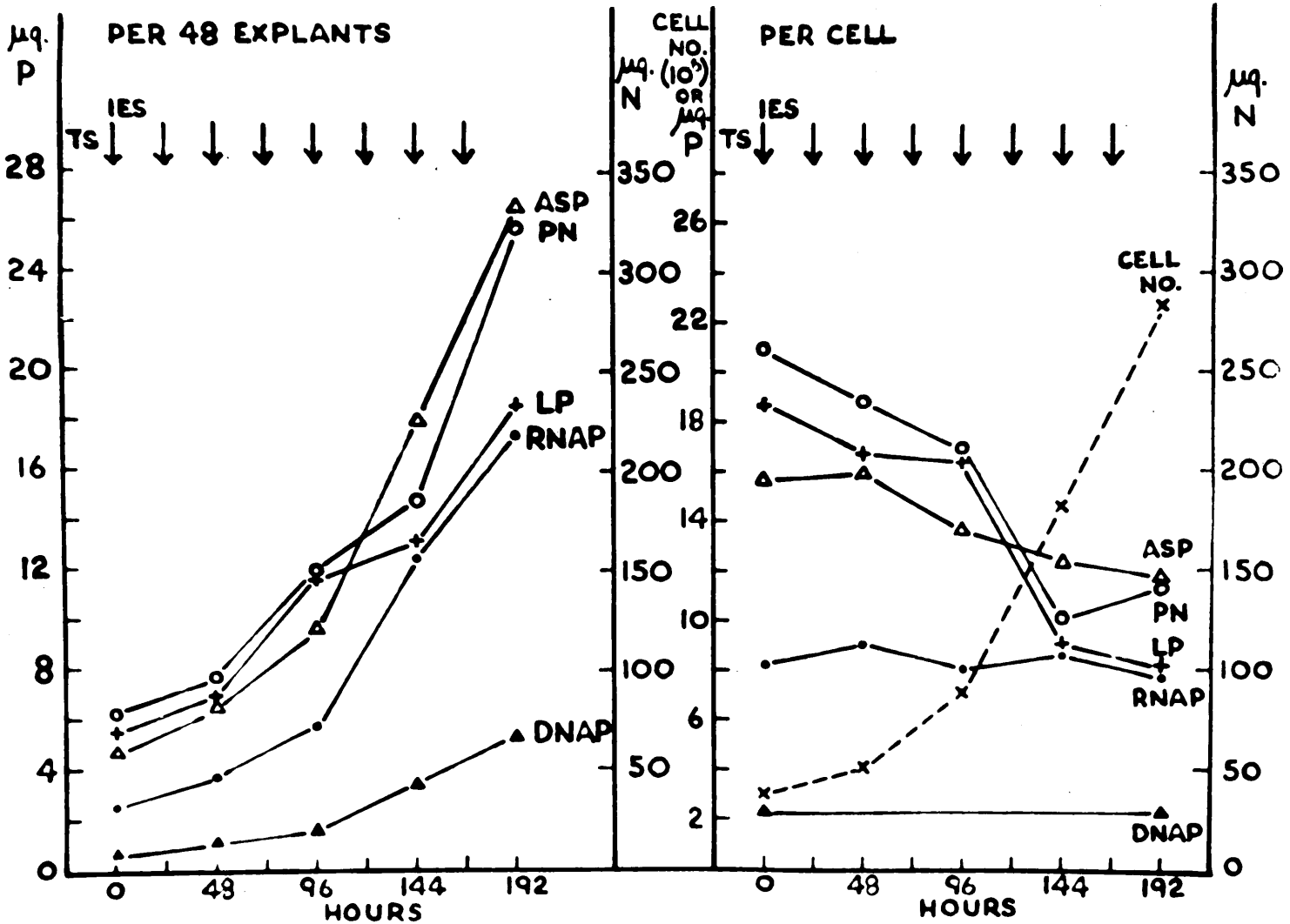
Fig. 11. Amounts per cell of constituents in 48 explants growing *in vitro*. Results are means obtained from a series of five tests, and all have been corrected for plasma blanks. Points along the ordinates show amounts per cell for the same constituents in the 12-day chick embryo heart from which the explants were obtained. Hours indicate time during which explants were in contact with TS or IES; they were treated with TS for 24 hours following each period of growth in IES.


PER CELL
TS
IES
μq P
μq N
28
24
20
16
12
8
4
0
300
250
200
150
100
50
0
0
48
96
144
192
240
HOURS
ASP
PN
LP
RNAP
DNAP

This, as had been found in preliminary experiments, greatly retarded the rate of growth, and produced only slight changes in composition. It gave, therefore, information on the general composition of the cells in explants which were brought to a 'resting state' at each stage of their growth. How little this operation influenced the change in cell number and composition of the actively growing explants can be seen in the results of an experiment in which the explants were still in contact with IES at the time of sampling. As before the amounts of material in 48 explants in contact with TS for 24 hours were taken as the basis for measuring subsequent increases; the results shown in Fig. 12 have been corrected for plasma blanks, which were kept at a minimum by repeated centrifugation of the IES at 3000 r.p.m. throughout the course of the test. On the left of Fig. 12 the final seven-fold increase in DNAP or cell number is the result of growth of the explants over the whole surface of the roller tube. Again the phase of rapid cell multiplication, in this test from 95 to 144 hours, involved a two-fold increase in cell number over 48 hours.

Changes in cell composition can be seen on the right of Fig. 12, where they can be compared with the increases in cell number represented by the dotted

Fig. 12. Chemical changes in 48 chick heart explants grown in vitro, but in this test (G 18) explants were not kept in contact with TS for 24 hours following their growth period in IES, as in G 17 (Fig.10). All results have been corrected for plasma blanks. Left section - amounts per 48 explants. Right section - amounts per cell based on DNAP content (as in Fig. 7). Unbroken lines - amounts of constituents; broken line - cell number.


µg. P
PER 48 EXPLANTS
IES
TS
ASP
PN
LP
RNAP
DNAP
CELL NO. (10³) OR µg. P
µg. N
HOURS
PER CELL
CELL NO.
µg. N
0
48
96
144
192

line. These follow the same general pattern of a steep decline in the amounts of PN and LP over the phase of rapid growth, and a close association between the ASP and RNAP of the cells. In the normal chick heart from 12 day embryos we have found that the RNAP content per cell is on the average 6.25 µg. In this test it had already risen to 8.2 µg. in the first 24 hours in TS, and it fluctuated in the region of this amount throughout the period in contact with growth-promoting medium. The highest value was 9.1 µg. at 48 hours, and the lowest 7.7 µg. at 192 hours. The trend is the same as before, but the IES has apparently prevented the decline in the RNAP per cell that was evident in explants brought to their 'resting state' in TS.

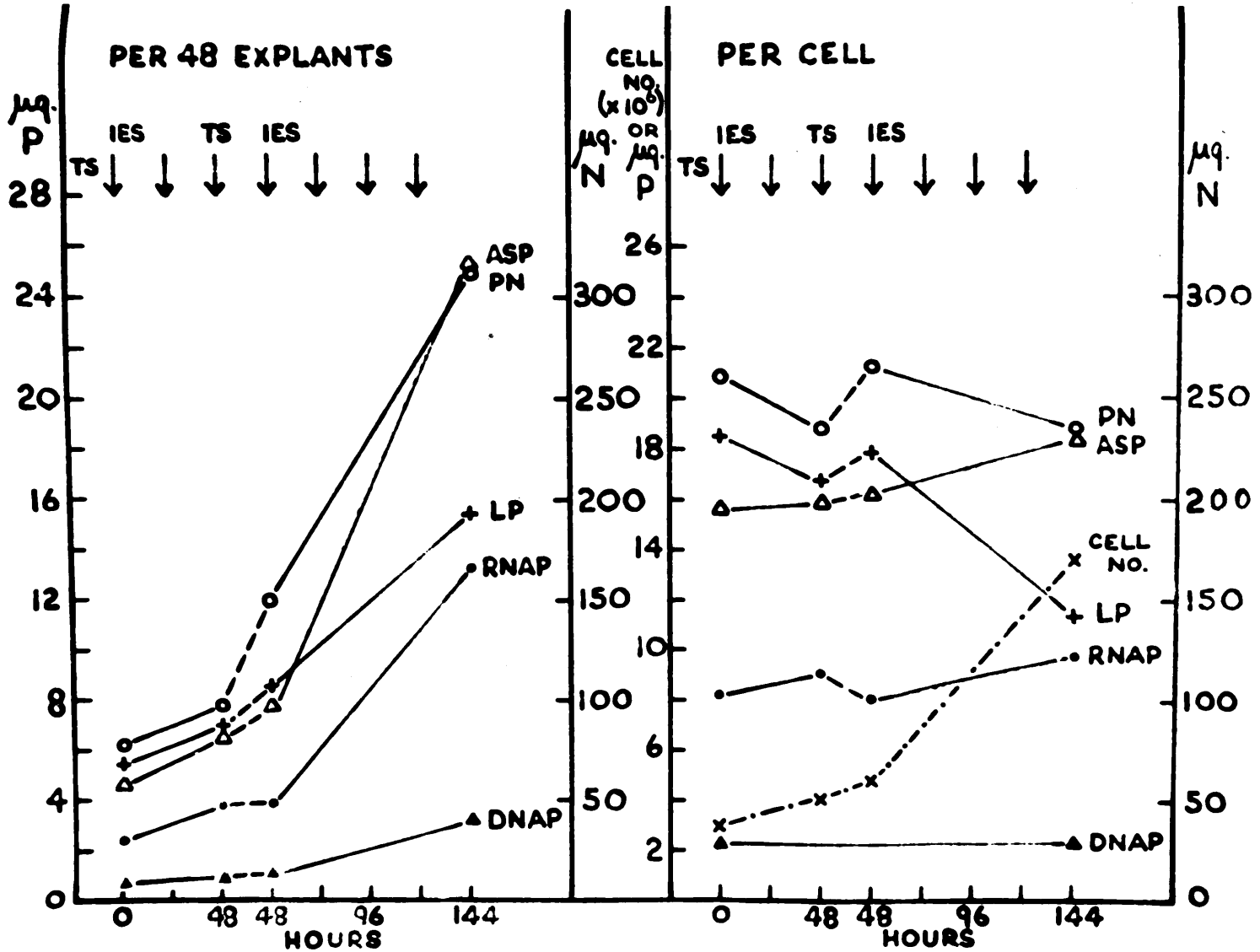
(h) Effects of Tyrode-serum on growing explants:

We have seen that there is little difference between the pattern of changes found in cultures sampled direct from IES and in those which remained in contact with TS for 24 hours after their growth phase. In both cases, high values for ASP and RNAP per cell were associated with rapid cell proliferation with the tendency for these to decrease when growth-promoting medium was replaced by TS. It was thought it desirable to investigate further the effect of

Uprode-serum on the cultures, and, in particular, to find whether their ability to grow would be impaired by keeping partly grown cultures in TS for 24 to 48 hours before allowing them to grow again in IES.

The changes per roller tube produced by keeping 48 hour explants in TS for a further 24 hours can be seen in the left of Fig. 13. The amounts of the various constituents increased slightly, PN showing a rather larger increase than the rest. When cultures treated in this way were given a further spell of 96 hours in contact with IES, their final values for all the constituents left no doubt that normal growth had continued in the second phase. For this tube and its control, which had spent an uninterrupted 144 hours in IES, the cell numbers were 13.6 and 14.5 millions respectively. The final RNAP and LP values were closely similar, but the ASP and PN were higher in the former than in the control. The right-hand section of Fig. 13 shows that the cell composition altered only slightly, if at all, during the 24 hour interval in TS. The fall in the RNAP per cell is perhaps significant by contrast with the increases in other constituents. As this slight change in composition occurred, the cell number rose from 4.1 to 4.8 million.

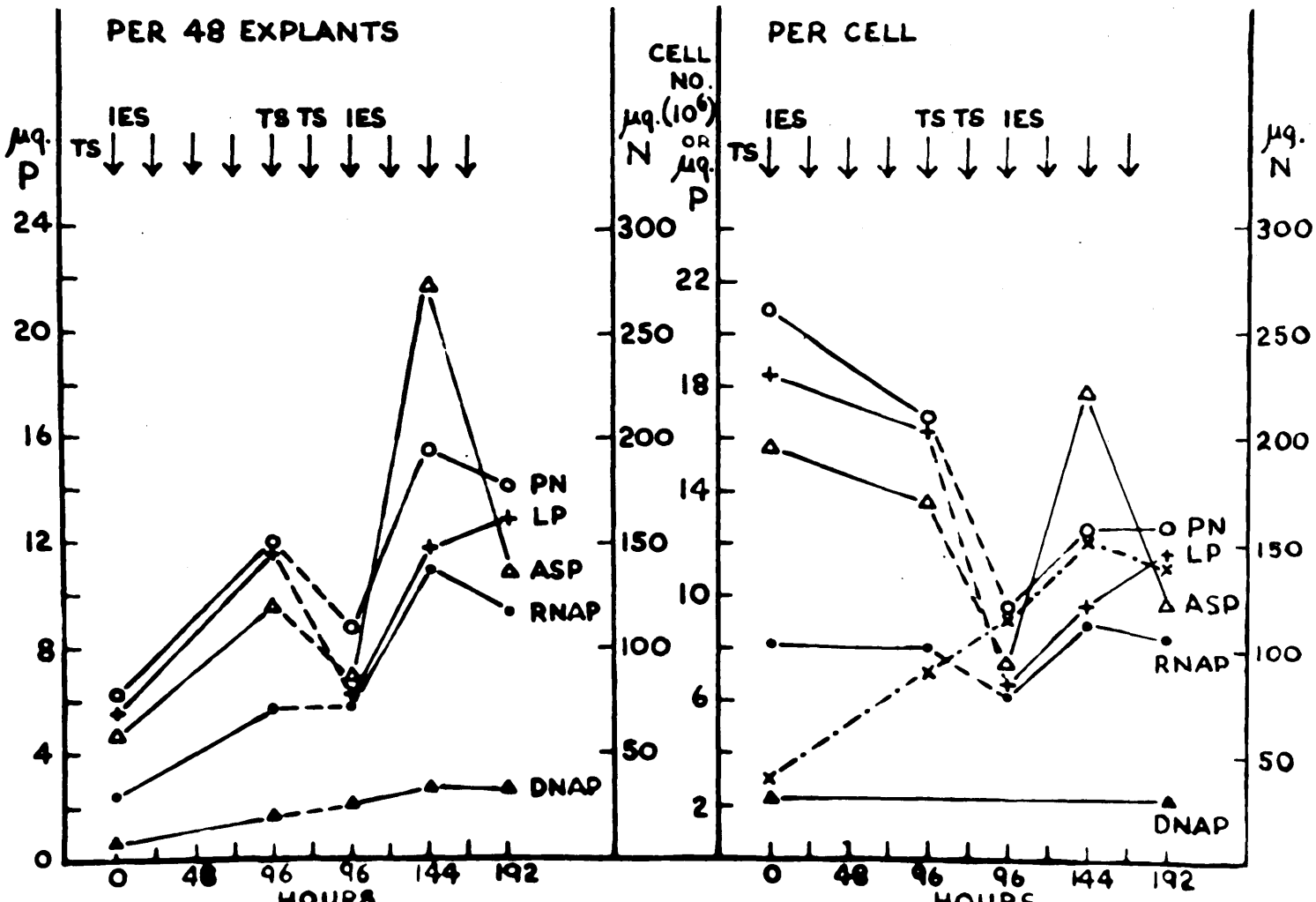
Fig. 13. Effect of keeping 48-hour growing chick heart explants in contact with TS at 37°C for 24 hours before resuming growth in IES. Left section - amounts per 48 explants corrected for plasma blanks. Right section - corrected amounts per cell. Unbroken lines show amounts of constituents after periods of growth in IES; broken lines show amounts after 24 hours in TS. Dotted line gives changes in cell number. Scale of hours indicates time in contact with IES.


PER 48 EXPLANTS
µg. P
TS
IES
TS
IES
28
24
20
16
12
8
4
0
ASP
PN
LP
RNAP
DNAP
0
48
48
96
144
HOURS
CELL NO. (x 10⁶) OR µg. P
µg. N
300
250
200
150
100
50
26
22
18
14
10
6
2
PER CELL
CELL NO.

The effect of keeping 96 hour explants in TS for a 48 hour interval was investigated in a similar experiment. On the left of Fig. 14 are shown the amounts per 48 explants at different stages of the test. During the two days in TS, only the DNAP and RNAP increased or remained unchanged; in contrast the amounts of PN, LP and ASP decreased significantly. Although the cell number shown on the right of Fig.14 increased from 7.1 to 9.2 millions in this time, it would have normally reached about 14 million had the cultures remained in contact with IES. Restoration of IES to the cultures produced a subsequent rise in cell number, but the final figures in the region of 11 to 12 million are much less than the 14.5 and 22.6 million found at similar stages in cultures taken from the same batch.

From the changes in cell composition, given on the right of Fig.14, it is clear that the cells at the end of the 48 hour interval contained about half the PN, LP and ASP that was present in the explant cell after 96 hours growth. However, in the cases of PN and LP, the same change (a fall in the amount of each per cell) occurred when the explants continued to grow in the normal manner in IES from 96 to 144 hours, the main difference being confined to the higher ASP and RNAP values in the rapidly growing cells. The return

Fig. 14. Effect of keeping 96-hour chick heart explants in contact with TS at 37°C for 48 hours before resuming growth in IES. Otherwise as for Fig. 13.


PER 48 EXPLANTS
PER CELL
IES
TS
TS TS IES
µq. P
µq. N
CELL NO. (10⁶) OR µq. P
PN
LP
ASP
RNAP
DNAP
HOURS
0
48
96
96
144
192
24
20
16
12
8
4
0
300
250
200
150
100
50
22
18
14
10
6
2

of the explants to growth-promoting medium caused slight increases in the amounts of the constituents per cell and resulted in limited growth.

3. INFLUENCE OF INSULIN ON GROWTH OF CHICK HEART EXPLANTS.

(a) Introduction: There is ample evidence from *in vitro* experiments to show that insulin promotes the synthesis of glycogen and fat in animal tissues (cf. Villee, Deane and Hastings, 1949; Chernick & Chaikoff, 1950). Although it is also generally accepted that insulin can have a stimulating effect on protein synthesis, the evidence has not been so decisive as the observations made in the case of glycogen and fat synthesis. *In vivo* experiments of Mirsky (1938, 1939), showing that insulin had a nitrogen sparing effect in normal, eviscerated and depancreatized dogs, suggested that it exerted a protein anabolic effect in animal tissue. His later work (Mirsky, 1939) led him to the view that the anterior pituitary acts through a pancreatic hormone, which might be identical with its growth hormone. Confirmation of the role of insulin as a promoter of protein anabolism, *in vivo*, has since come from other authors (cf. Lotspeich, 1949; and Forker, Chaikoff, Entenman & Tarver, 1951) but the question of its identity as a 'growth hormone' has so far not been settled by such

experiments.

The tissue culture technique offers a direct method of studying this problem. In the only extensive work which has so far been reported, Latta & Bucholz (1939) found that insulin increased the growth of chick fibroblasts, but only when the insulin concentrations were abnormally high at 33 units per ml. of medium. Any increase visible at one-tenth that concentration was very slight, and the possibility remained that the growth-promoting effect was not the result of the action of insulin as a hormone.

The experiments described here have been designed to provide sufficient measurements to allow the statistical analysis of results. As it was established that corrections for plasma blanks did not materially alter the pattern of results during growth, it was possible to eliminate these in making comparisons between cultures under test with insulin and the control cultures. The number of explant tubes was accordingly increased, and they were taken for chemical analysis in batches of six after 96 and 144 hr. of growth.

The growth-promoting medium was embryo extract mixture (EES) containing 80-100 mg. N per ml. It was divided into two batches at the beginning of each test, and sterile insulin in Tyrode solution

added to one of these in sufficient volume to give a final concentration of 2-3 units insulin per ml. (The original solution of insulin supplied by Messrs. Boots was free from preservative, and was diluted with an equal volume of double strength Tyrode solution). Allowance was made for the amount of protein added (24 units per mg. protein), and the normal EES was diluted with Tyrode solution to bring it close to the same final N concentration as the insulin-EES. As before, the growth-promoting extract in the roller tubes was replaced by a fresh lot every 24 hours throughout growth.

(b) Effect of Insulin on cell proliferation:

The cell number (Table 15) has been calculated from the amounts of DNAP per 48 explants (Table 14). From an initial mean value of 2.5 million, the final figures at 144 hr. were 8.0 million for cells growing in normal EES, and 11.8 million for cells under the influence of insulin, this last figure representing an increase of nearly 50% above the normal value. The results of applying the 't' test (Fisher, 1936) confirm that the mean increase in cell number was highly significant. A similar difference between insulin-treated explants and controls at 96 hr., did not prove to be statistically significant.

TABLE 14.

Amounts per 48 explants of cell constituents in insulin-treated and control explants.

Explants	Test	Age	ASP	LP	RNAP	DNAP	PN
		hrs.	µg.	µg.	µg.	µg.	µg.
48	Initial	0	2.7	4.4	1.64	0.58	66
48	Control	96	15.1	5.5	4.96	1.51	127
	Insulin	96	15.9	8.0	7.92	2.14	179
n=6	"t"=	-	-	2.16	2.11	1.44	2.92
48	Control	144	37.0	6.8	5.48	1.88	186
	Insulin	144	21.7	10.8	10.34	2.78	263
n=10	"t"=	-	1.52	4.39	6.84	3.03	3.37

When n=6, the "t" value corresponding to P=0.05 is 2.447

When n=10, the "t" value corresponding to P=0.05 is 2.228

TABLE 15.

Cell number and amounts per cell of constituents in insulin-treated and control explants.

Explants	Test	Age	Cell No. ($\times 10^6$)	ASP	LP	RNAP	PN
48	Initial	0	2.5	11.1	17.8	6.63	270
48	Control	96	6.43	23.5	8.55	7.71	198
	Insulin	96	9.10	17.4	8.78	8.70	196
n=6	"t"=	-	1.44	1.86	-	0.93	-
48	Control	144	8.0	46.3	8.5	6.85	232
	Insulin	144	11.8	18.3	9.1	8.75	222
n=10	"t"=	-	3.03	3.12	1.14	2.46	-

When n=6, the "t" value corresponding to P=0.05 is 2.447

When n=10, the "t" value corresponding to P=0.05 is 2.228

(c) Effect of Insulin on the total synthesis of cell constituents:

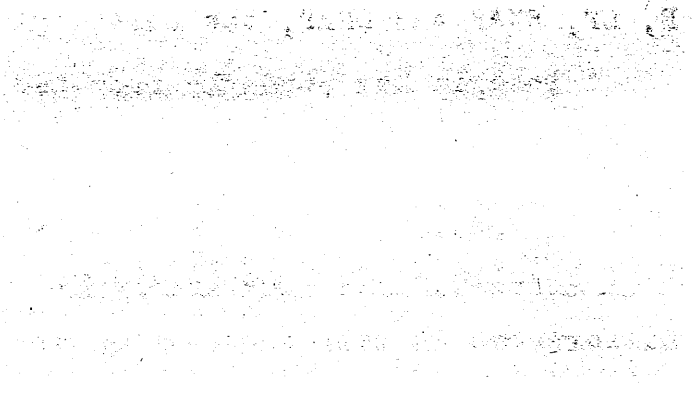
Table 14 shows the total amounts per 48 explants of various cell constituents. At 96 hr. only PN is seen to have been synthesized to a significantly greater extent as a result of the presence of insulin, but at 144 hr. the amounts of LP, RNAP, and PN are all very much greater in the insulin-treated explants. In contrast, the ASP per 48 explants is rather lower than normal after growing in insulin-EES for 144 hours.

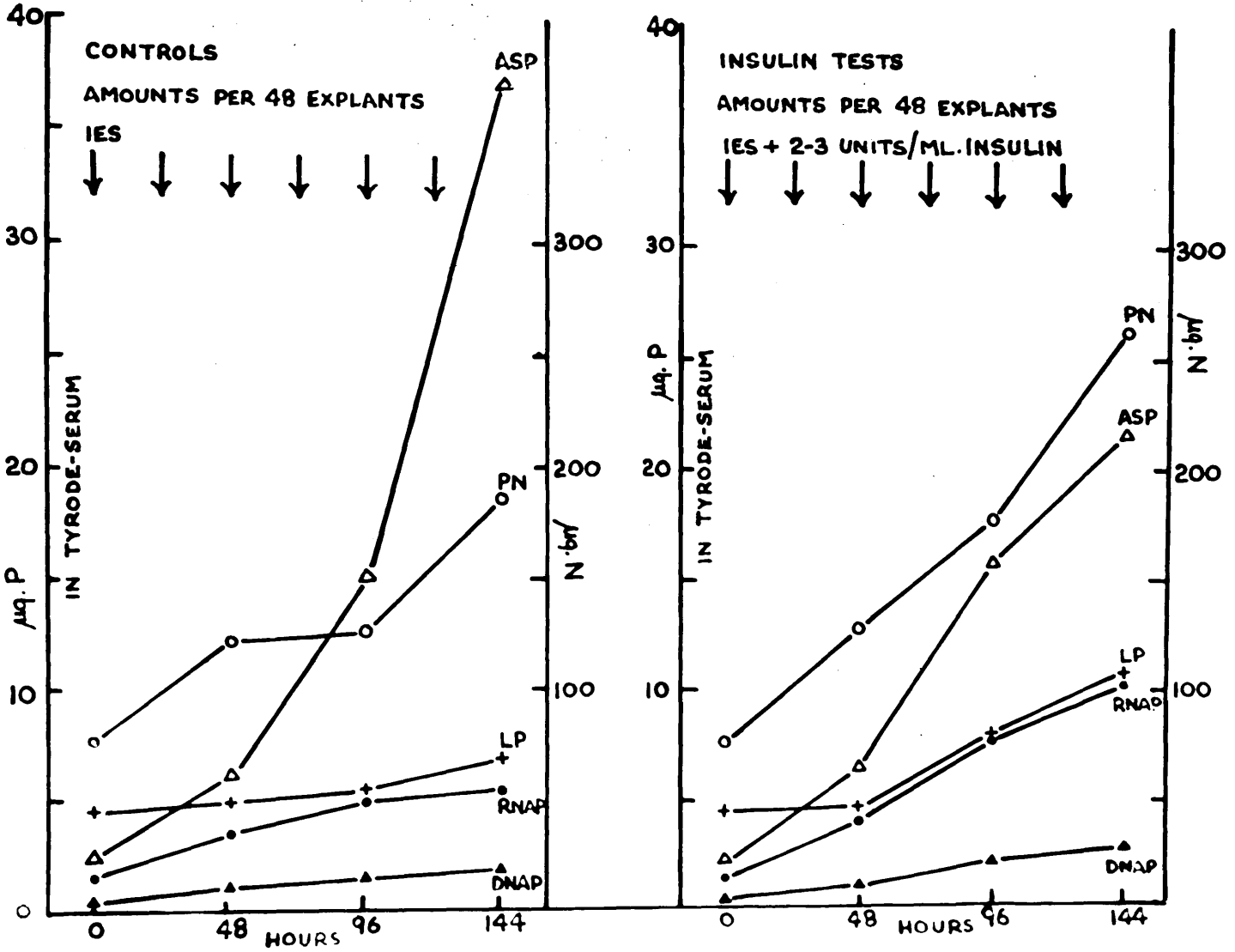
When these results and the means of 4 observations made on 48 hr. explants are compared graphically in Fig. 15, the steep upward rise of the ASP in normal cultures (when not corrected for plasma blanks) is strikingly reduced by the action of insulin. But with PN, LP, RNAP and DNAP, the curves give the impression that insulin has strongly promoted the accumulation in the explants of these four constituents.

(d) Effect of Insulin on Cell Composition:

Differences in cell composition are analysed statistically in Table 15. At 144 hr. the RNAP content per cell is distinctly greater in the insulin-treated explants, while the ASP per cell is very much less than normal. The amounts of PN and LP in the cells are

Fig. 15. Amounts per 48 explants of cell constituents in insulin-treated and control explants.

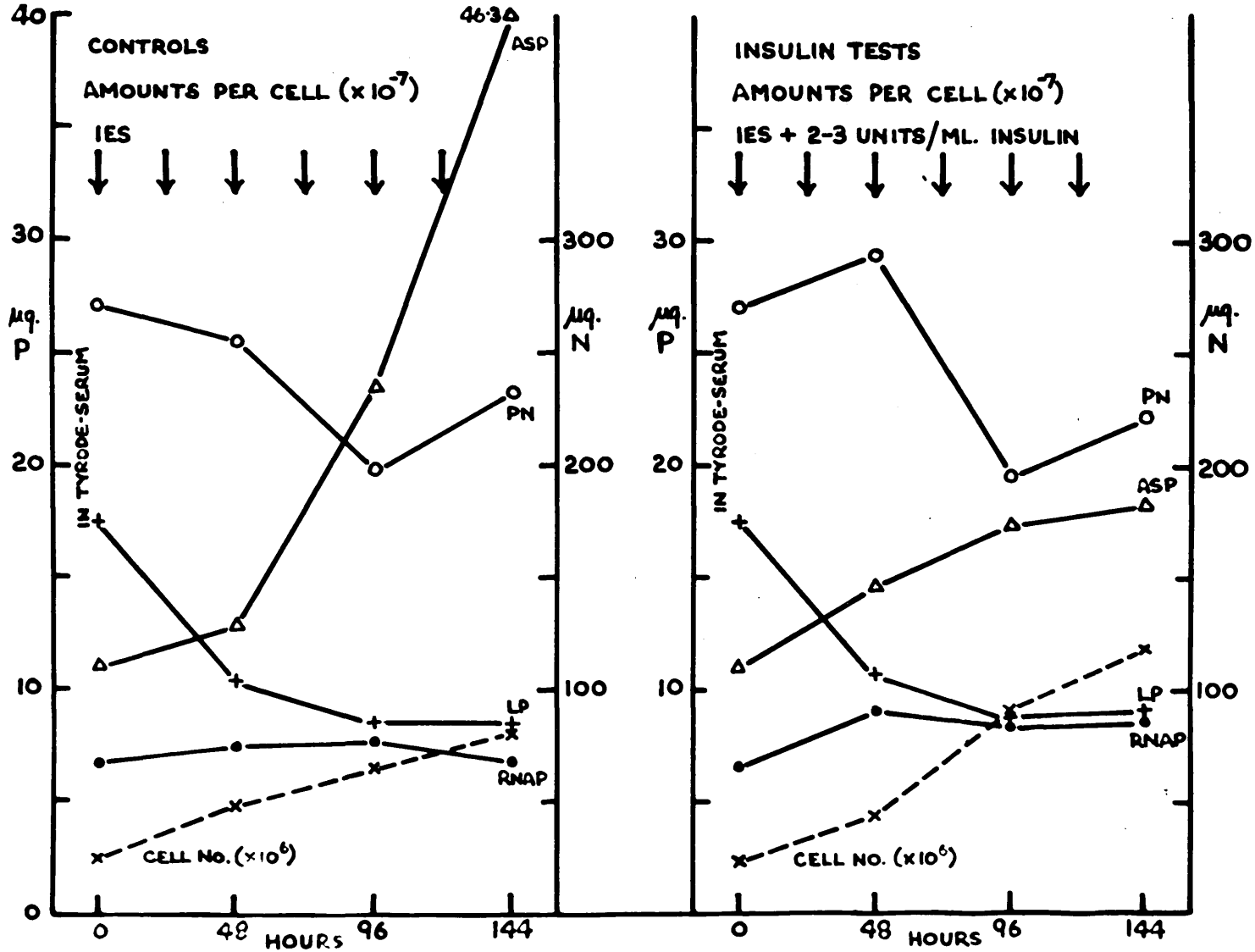



CONTROLS
AMOUNTS PER 48 EXPLANTS
IES
ASP
PN
LP
RNAP
DNAP
μg. P IN TYRODE-SERUM
N·μg
HOURS
0
48
96
144
10
20
30
40
100
200
300
INSULIN TESTS
AMOUNTS PER 48 EXPLANTS
IES + 2-3 UNITS/ML. INSULIN

apparently not influenced by insulin. There was no significant difference in the means for the two groups at 96 hr., but even at that stage the same trends were evident.

When these results are seen graphically (Fig. 16) there is no doubt about the similarity in the pattern of changes of PN and LP in normal and insulin-treated explants. The essential differences produced by insulin are the increased cell number, the high values of RNAP per cell maintained throughout growth, and the very much smaller value of ASP per cell at the end of the growth period. It may be added that the pattern of changes for the normal explants in these tests is closely similar to that obtained in previous results, when no correction was made for amounts in plasma blank tubes.

Fig.16. Cell number (broken line) and amounts per cell of constituents in insulin-treated and control explants.


CONTROLS
AMOUNTS PER CELL (x10⁻⁷)
IES
46·3
ASP
PN
LP
RNAP
CELL NO. (x10⁶)
µg. P
IN TYRODE-SERUM
µg. N
HOURS
INSULIN TESTS
AMOUNTS PER CELL (x10⁻⁷)
IES + 2-3 UNITS/ML. INSULIN
0
48
96
144
10
20
30
40
100
200
300

C. DISCUSSION

If we consider first the changes in cell number of 48 explants as determined from the total DNAP content of the tissues, we find the following characteristic features of growth in vitro. There is in the period immediately following the planting of the cultures an unavoidable loss of the main cell constituents, and from the decreases in DNAP we can deduce that from one to two-thirds of the cells are lost when the cultures remain for 24 hours in Tyrode-serum solution. The losses are, not surprisingly, greatest in the smaller explants, since these must be more extensively damaged by the cutting process. It is also clear that such losses are only slightly diminished by keeping the explants in contact with growth-promoting medium from the time of planting.

In all tests in which relatively large pieces of tissue were used as explants, any increases of DNAP (or cell number) were small in comparison to those of RNAP, and of a temporary character (Fig.3). Only by reducing the explant size could the DNAP increase significantly, and remain at its new high level in the final equilibrium stage, during which there is an even balance between cell formation and cell destruction.

The virtual elimination of the plasma in

the final procedure has in some way altered the ratios of RNAP:DNAP found during the growth phase of small explants. The values for this ratio are much higher in the case of explants grown in plasma clots, but common to both techniques is the lag in the increase of DNAP during the first 48 or 72 hours. It is also evident that in both cases the RNAP:DNAP ratio (and hence the amount of RNAP per cell) reaches its peak value during the phase of rapid cell proliferation between 48 and 144 hours.

When, as in the earlier technique, 24 small explants are grown in plasma clots, the largest increase in DNAP over the amount in the explants at zero time is 160% (Fig.4). With the modified technique using 48 explants per tube and little or no plasma, the increases in DNAP (or cell number) over the same period of 6 days are of the order of 500%. At this high growth rate in cultures of 12 day chick embryo heart explants, the cell number invariably doubles in each 48 hour period between 48 and 144 hours, and it is perhaps significant that this is the same as the growth rate of cells of the chick embryo heart between the 12th. and 14th days of incubation (Section IV). Similar growth rates are recorded for chick heart fibroblasts in earlier studies summarized by Fischer (1946).

The high rate of cell multiplication certainly depends on the provision of an adequate growth-promoting medium; it was never exceeded in the presence of IES containing 4.5 to 6 mg. of protein, that is roughly 6 to 10 times the amount of protein present in 48 explants. On the other hand, cell multiplication was greatly retarded when the explants were in contact with serum containing about 3 mg. of protein for periods of 24 and 48 hours. In one test (Fig.14) instead of the normal increase in cell number of 100% over 48 hours, only a 30% increase was obtained in the presence of Tyrode-serum. This occurred after the cultures had been growing in IES for 96 hours, and had reached the stage when their growth rate was normally at its peak.

The few results obtained for explants of different sizes (i.e. within the limits required to obtain appreciable growth) show that the number of cells present when growth is complete or nearly so, is directly proportional to the number in 48 explants at the start of the growth period. There have never been increases greater than six or sevenfold, but this limit was reached whether the initial explants contained a total of 1 million or 3 million cells. The time required for the cultures to grow to their

maximum size varied between 6 and 10 days, but since these tests were carried out at different times with different batches of material it is not possible to decide how far the initial size affected the rate of growth. Hull and Kirk (1950 a,b,c) have raised some doubts about earlier observations summarised by Fischer (1946), which indicated that the larger the initial explant, the greater was the final area. This conclusion was also reached by Brues, Rathbun and Cohn (1944) who noted much less migration and mitosis in small explants than in larger ones. Although in these earlier studies the relationship between cell number and the area of the explant has not been precisely defined, the present results support the general concept that the number of cells initially in the explant determine the final number in fully grown explants.

It would be of some value at this stage to discuss how far changes in the chemical composition of cells growing in vitro will enable us to understand the chemical events which lie behind the multiplication of cells. It seems that an answer to this question should come from the study of the changing cell composition under conditions in which a large proportion of the cells are actively dividing without at the same time undergoing a permanent change in composition.

This requires a system in which the cells in the fully grown explants are closely similar in composition to the original cells, since this will ensure that any changes in composition during growth will be part of the chemical process leading to cell division.

Such conditions are in fact found in the roller tube cultures of chick heart explants, in which the final cell composition is not significantly different from that of the cells in the original fragments of heart tissue (Table 11). In the earlier stages of growth, too, the 70,000 to 150,000 cells in each of 48 explants double their number in a two-day interval. As the average time between one mitotic division and the next has been estimated as 10 hours (Fischer, 1946), the proportion of cells approaching or undergoing mitosis must be appreciable in the rapid phase of growth. A detailed study of explants from 8 day chick heart by Tompkins, Cunningham and Kirk (1947) showed 20 to 160 mitotic cells per 1000 total cells in 24 and 48 hour cultures according to the region of the explant examined. In view of the relatively short duration of the actual mitosis, it is safe to assume that the proportion of cells whose composition is changing prior to division, will be appreciably larger than these figures for the proportions of

mitotic cells might suggest. There is, consequently, good reason to conclude that the changes in composition of the cultures are related to the chemical processes leading to cell division.

The onset of growth is always accompanied by large increases in the amounts of protein and phospholipid per cell in the 24 hours following planting. The corresponding changes in the ASP and RNAP depend to a much greater extent on the nature of the medium; with Tyrode-serum their amounts per cell remain unaltered or increase slightly, but with IES the effect is to increase these amounts significantly. As the cultures enter their rapid growth phase after 48 hours, the protein and phospholipid content of the cells begin to fall and reach their original levels at 144 hours or later. In contrast, the ASP and RNAP per cell both remain at their high levels throughout the phase of rapid growth between 48 and 144 hours. All constituents return to near their levels in the original cells of the heart tissue in the final slow phase of growth after 144 or 192 hours.

From this consistent pattern of changes we can draw two conclusions. First, there is in the phase before rapid cell division a two to threefold increase in the average cellular content of protein and phospholipid. This is strikingly supported by

the determinations of average cell mass in similar cultures by Tompkins, Cunningham and Kirk (1947). They found that the average cell mass in the peripheral growth areas of the explants was higher in 24 and 48 hour cultures than in cultures at 72 and 96 hours, and they concluded that these larger cells were two to four times the mass of cells in the centre of the explants. When it is remembered that the proportion of cells undergoing mitosis is also much greater in 24 and 48 hour cultures, there seems little doubt that the increased cell sizes are the result of increased protein and phospholipid per cell prior to division. For this reason, and because of the relatively slow synthesis of DNAP in the first 48 hours of growth, it is concluded that the protein and phospholipid components of the cells are synthesized before the DNA of the chromosomes.

The second characteristic feature is the association between ASP and RNAP and the relatively high amounts of these constituents in the cells during the phase of rapid cell multiplication. The amounts of both per cell are still maximal at 96 hours, when there is already an appreciable decline in the protein and phospholipid content of the cells. This seems to suggest that ASP and RNAP have some part to play in the process of cell division which is distinct

from the generally accepted association between RNA and protein synthesis; for, if we compare the percentage increases at 192 hours over the amounts present per 48 explants after 96 hours in IES (Fig.12), we find that the protein increased by 114%, the RNAP by 207% and the DNAP by 231%. In other tests in which the cultures spent 24 hours after growth in Tyrode-serum, there was very little protein (per 48 cultures) synthesized after 96 hours (Fig.10), while the RNAP continued to increase as more DNA was synthesised. At the same time there was a reduction in the average RNAP content of the cells in this later phase which coincided with the lower rate of cell multiplication.

Further evidence which suggests that RNA is linked in some way to the synthesis of DNA comes from the experiments in which rapid growth was halted by keeping the cultures in Tyrode-serum for 24 and 48 hours. In both cases there was little or no increase in the total amounts of RNAP per 48 cultures, but there were 15 and 30% increases in the total DNAP values. As a result we see (Figs. 13 and 14) that the synthesis of this DNA was accompanied by corresponding reductions in the average amounts of RNAP per cell. This, of course, does not necessarily involve a quantitative conversion of one to the other, but the fact that only the RNAP consistently behaved in this way suggests

that the accumulation of RNA and DNA in the cells are in some way interrelated. This view does not conflict with their possible roles in protein synthesis, since all the evidence from studies with tracer elements indicates that the synthesis is accompanied by an intensification of the metabolic turnover of the nucleic acids rather than a process of transformation.

There is one puzzling aspect of the chemical composition of the fully grown explants. It is generally accepted that the growth of cells in vitro is accompanied by a return to a more generalised cell type (Willmer 1945), or by a process of 'dedifferentiation' (Fischer 1946). In the studies on the chemical development of chick embryo organs (Davidson and Leslie, 1950a, b; 1951) it has been found that differentiation is accompanied, amongst other things, by a striking increase in the protein content of the cells, which suggests that this might be characteristic of cells which are developing to their adult or specialised forms. In a more recent paper, reviewing studies on the chemical changes in induced tumours, evidence has been presented to show that tumour cells contain much less protein than the corresponding cells in normal tissue (Davidson and Leslie, 1950b). This, too, can be understood as part of the chemical change

involved in the return from the normal adult cell to a more primitive form. But there is not a similar change in the composition of the chick heart cells cultivated in vitro, as might be expected from the concept of their 'dedifferentiation'. It is quite clear from the present results that the final cell composition is not significantly different from that of the cells in the 12 day embryo heart, even after a seven-fold increase in their number in vitro, and that the decreasing amounts of protein per cell are unconnected with 'dedifferentiation' as has been suggested elsewhere, (Davidson & Leslie, 1950 a,b).

Some aspects of the action of insulin on the growing explants should be stressed. Insulin can bring about an increase in cell number (as judged by total DNAP synthesis) to 50% above the normal amount in the course of 6 days growth. It promotes the synthesis and accumulation of PN, RNAP and LP, but produces explants which contain rather less ASP than normal. Cells under the influence of insulin contain on the average less ASP and more RNAP than those growing under normal conditions, but the amounts per cell of LP and RNAP are unaltered.

The ability of insulin to increase the RNAP content of cells does not appear to be

confined to the rapidly proliferating fibroblasts, as increased RNA concentrations have been produced in the nerve cells of the rabbit spinal cord during treatment with insulin (Hyden, 1947; Hochberg & Hyden, 1949). Malononitrile also produced this effect, and because it inhibited the cyanide-sensitive enzyme systems, the Swedish authors suggested, perhaps rather unconvincingly, that the action of insulin was, likewise, the result of impaired oxidative metabolism in nervous tissue. However, the increased cell proliferation in the chick heart explants rather indicates that insulin has a general stimulating effect on cell metabolism, promoting the synthesis of phospholipid and protein, as well as that of ribonucleic acid.

This view is supported by recent evidence, e.g. the observation that insulin stimulated respiration in preparations of brain tissue when sufficient succinate was available in the medium (Goranson & Erulkar, 1949). It is well established, too, that insulin increases the utilization of glucose; for example, during glycogen synthesis in the isolated rat diaphragm muscle, glucose utilization is increased

to a greater extent than can be accounted for by the formation of glycogen (cf. Haugaard, Marsh & Stadie, 1951). Finally, there is the interesting observation that insulin increased the rate of oxidation of glucose by Pasteurella pestis without the insulin itself being destroyed by the organism (Levine, 1950).

One clear result of this action of insulin is the increased formation of phospholipid and RNAP at the expense of the ASP in the culture cells. Much the same effect is reported by Haugaard, Marsh & Stadie (1951), who find that insulin significantly increases ester phosphate in rat diaphragm muscle while glycogen is being synthesised. In view of these effects, it seems likely that an essential step in the general stimulus given by insulin to cell metabolism is the increased formation and utilization of energy-rich phosphate bonds, as is suggested by the work of Sachs (1945) and of Goranson & Erulkar (1949) showing that insulin stimulates the uptake of ^{32}P in phosphocreatine and ATP in muscle and in brain homogenates. Whatever may be revealed in future work on the nature and action of the anterior pituitary 'growth hormone',

it is quite certain already that insulin is very well qualified to act as a growth-promoting agent in the way originally envisaged by Mirsky (1939).

C SECTION IV

CELL DEVELOPMENT IN CHICK EMBRYONIC TISSUES

A. INTRODUCTION

Of the numerous biochemical studies which have been made on the developing chick embryo the great majority have been concerned with chemical changes occuring in the embryo as a whole (Needham 1931,1942). By comparison only a few investigations have been made on the chemical changes within individual organs during development. This is unfortunate, since it is frequently difficult to assess the significance of the results derived from so complex a system as the whole embryo, particularly when changes are described in terms of concentrations per unit weight of tissue. Not only are the various organs developing in different ways and at different rates during the various phases of embryonic life, but their chemical composition also changes as they develop towards their adult functional state.

Even when the organs are studied separately, difficulties arise in the interpretation of results, especially when analytical figures are expressed in terms of concentration of a constituent per unit weight of tissue, since the weight itself refers to a constantly changing complex of variables. There

are, furthermore, the errors introduced by biological variations as instanced by the problem of deciding embryonic age (Levy & Palmer 1943).

Some of these difficulties may be overcome by following the chemical development of tissues in terms of the changes in cell number and cell composition. This has been done in the study of brain, heart, liver and muscle in chick embryos, using the procedure already applied to the measurement of growth in tissue cultures. Again, it is based on the assumption that the DNAP per cell in these tissues remains constant throughout growth at 2.35×10^{-7} µg., the average amount common to both chick embryo and fowl tissues (cf. Davidson, Leslie, Smellie & Thomson, 1950).

The use of DNA instead of wet or dry weight as a standard of reference has several advantages in biochemical studies on embryonic growth. The weight of an organ can rise by increase in cell number or cell size, or by a combination of both. By reference to DNA the two processes can be distinguished. When the weight of the tissue is related to the amount of DNA per cell, it becomes possible to follow changes in average cell mass throughout development. These changes can, in turn, be correlated with the determinations of cell number and cell composition.

Another important advantage is that results

expressed as amounts per cell of any constituent are independent of the proportions of the other constituents of the tissue. This also applies to the determination of growth rates of cell constituents relative to that of DNA. In the allometric method originally proposed by Teissier (1931) and Needham (1932,1934) growth rates were based on the wet or dry weight of the whole, and their significance was to some extent obscured because the constituent was part, and sometimes an important part, of the complex of variables which represent the weight. On the other hand, when based on DNA, the relative growth rates show quantitively how a constituent varies with increasing cell number over distinct phases of embryonic development.

B. RESULTS

(a) Methods of expressing results:

It is customary to express the results of the chemical analysis of any tissue in terms of the absolute amounts of each component per organ or of the concentrations per unit weight of wet or dry tissue. This has been done in the first two sections of Tables 16-19 for chick embryo brain, heart and liver, but it has not been possible to give the absolute amounts per organ for skeletal muscle, since irregular samples of this tissue were taken rather than the whole organ. The third section of each table, by relating the wet weight and amounts of ASP, LP, RNAP, non-protein nitrogen (NPN), and PN to the constant value of DNAP, shows how the cell mass and cell composition vary during development.

Comparison of the results expressed as concentrations per unit weight of fresh tissue with the absolute amounts per cell, reveals that there are substantial differences in trend between the two sets of figures during the growth of the brain, liver and muscle. For example, in brain (Table 16) the concentration of ASP decreases during development, while at the same time the amount per cell actually doubles. The protein concentration increases by about 40% in brain tissue during the embryonic period, whereas

TABLE

COMPOSITION OF

ASP = acid soluble phosphorus; LP = lipid phosphorus; RNAP = ribonucleic acid phosphorus;

Age (days)	Number of samples	Mean wt. of brains (mg/organ)	ASP	LP	RNAP	DNAP	NPN	PN	ASP	LP
			μg/organ						μg/100 mg	
8	12	56	60	43	28	16	168	400	107	77
10	20	107	107	80	49	20	231	786	100	75
	16	177	157	129	65	23	371	1190	89	73
13	12	241	207	190	96	30	496	1636	86	79
	16	333	274	286	125	38	620	2115	82	86
	14	539	470	526	195	53	1370	4098	87	98
	14	666	532	734	269	68	1785	4476	80	110
Hatching	3	816	650	1125	371	94	1996	8295	80	138
2 days after Hatching	3	822	630	1220	434	86	2112	9560	77	148

TABLE

COMPOSITION OF

Abbreviations

Age (days)	Number of samples	Mean wt. of hearts (mg/organ)	ASP	LP	RNAP	DNAP	NPN	PN	ASP	LP
			μg/organ						μg/100 mg	
8	12	4	4	3	2	0.7	6	35	100	75
10	20	9	9.5	5.3	4.8	1.7	16	76	105	59
11	8	13	13	8	7	2.5	26	100	100	62
13	18	36	38	23	17	6	113	288	106	64
14.5	16	68	61	41	32	12	223	446	90	60
17.5	14	133	137	94	71	29	367	1350	103	71
19.5	14	174	158	126	83	39	351	1738	91	72
Hatching	3	237	186	165	116	48	570	2610	78	70
2 days after Hatching	3	310	261	236	118	59	1121	4139	84	76

16.

CHICK EMBRYO BRAIN

DNAP = deoxyribonucleic acid phosphorus; PN = protein nitrogen.

RNAP	DNAP	NPN	PN	Cell mass $mg \cdot 10^{-7}$	ASP	LP	RNAP	DNAP	NPN	PN
fresh tissue					$\mu g \cdot 10^{-7}$/cell					
50	29	300	715	8.2	8.8	6.3	4.1	2.35	24.6	59
46	19	216	735	12.6	12.6	9.4	5.8	2.35	27.1	93
37	13	210	672	18.1	16.0	13.2	6.6	2.35	37.9	121
40	12	206	680	18.9	16.2	14.9	7.5	2.35	38.8	128
38	11	186	635	20.6	16.9	17.7	7.7	2.35	38.3	131
36	10	254	760	23.9	20.8	23.4	8.6	2.35	60.5	182
40	10	268	672	23.0	18.4	25.4	9.3	2.35	61.5	155
45	12	245	1015	20.4	16.2	28.1	9.3	2.35	50.0	207
53	10	257	1160	22.4	17.2	33.3	11.8	2.35	57.5	261

17.

CHICK EMBRYO HEART

as in Table 16

RNAP	DNAP	NPN	PN	Cell mass $mg \cdot 10^{-7}$	ASP	LP	RNAP	DNAP	NPN	PN
fresh tissue					$\mu g \cdot 10^{-7}$/cell.					
50	17	150	870	13.4	13.4	10.1	6.7	2.35	20.2	117
53	19	178	845	12.4	13.1	7.3	6.6	2.35	22.1	105
54	19	200	769	12.2	12.2	7.5	6.6	2.35	24.4	94
47	17	314	800	14.1	14.9	9.0	6.7	2.35	44	113
47	18	328	655	13.3	11.9	8.0	6.3	2.35	44	87
53	22	276	1015	10.8	11.1	7.6	5.7	2.35	30	109
48	22	202	1000	10.5	9.5	7.6	5.0	2.35	21	105
49	20	240	1100	11.6	9.1	8.1	5.7	2.35	28	128
38	19	362	1336	12.3	10.4	9.4	4.7	2.35	45	165

TABLE

COMPOSITION OF

Abbreviations

Age (days)	Number of samples	Mean wt. of livers (mg/organ)	ASP	LP	RNAP	DNAP	NPN	PN	ASP	LP
			μg/organ						μg/100 mg	
8	12	11	15	10	13	3	35	118	136	91
10	20	22	28	22	25	6	70	342	127	100
11.5	16	49	57	47	49	10	140	583	116	96
13.5	10	117	139	122	126	26	421	1855	119	104
15	6	215	253	223	185	51	—	3015	118	104
17.5	10	392	456	418	372	80	1678	6553	116	107
19.5	16	546	505	573	498	122	1888	7743	93	105
Hatching	3	872	660	870	788	163	2720	15040	75	100
2 days after Hatching	3	1278	893	1303	1133	240	4700	24580	70	102

TABLE

COMPOSITION OF

Abbreviations

Age (days)	Number of samples	ASP	LP	RNAP	DNAP	NPN	PN
		μg./100 mg. fresh tissue					
11.5	12	84	40	45	21	198	410
13.5	22	105	57	59	25	282	795
15	12	82	39	41	19	206	645
17.5	12	85	47	47	20	235	967
19.5	18	85	49	47	17	270	1156
Hatching	3	98	47	36	12	352	1466
2 days after Hatching	3	107	60	38	14	417	1920

18

CHICK EMBRYO LIVER

as in Table 16

RNAP	DNAP	NPN	PN	Cell mass mg·10⁻⁷	ASP	LP	RNAP	DNAP	NPN	PN
fresh tissue					μg.10⁻⁷/cell					
118	27	318	1070	8.6	11.7	7.8	10.2	2.35	27.4	92
114	27	318	1556	8.6	11.0	8.6	9.8	2.35	27.4	134
100	20	286	1190	11.5	13.4	11.0	11.5	2.35	32.9	137
108	22	360	1584	10.6	12.5	11.0	11.4	2.35	38.0	167
86	24	—	1430	9.9	11.7	10.3	8.5	2.35	—	139
95	20	428	1674	11.5	13.4	12.3	10.9	2.35	49.3	193
91	22	346	1420	10.5	9.7	11.0	9.6	2.35	36.4	149
90	19	312	1720	12.6	9.5	12.5	11.4	2.35	39.2	217
89	19	368	1920	12.5	8.8	12.8	11.1	2.35	46.0	241

19.

CHICK EMBRYO MUSCLE

as in Table 16

Cell mass mg·10⁻⁷	ASP	LP	RNAP	DNAP	NPN	PN
	μg.10⁻⁷/cell					
11.0	9.2	4.4	4.9	2.35	21.8	45.1
9.5	10.0	5.4	5.6	2.35	26.8	75.5
12.1	9.9	4.7	4.9	2.35	24.9	78
11.8	10.1	5.6	5.6	2.35	27.7	114
14.2	12.0	7.0	6.6	2.35	38.3	164
20.2	19.7	9.4	7.3	2.35	71.0	296
16.9	18.1	10.1	6.4	2.35	70.5	324

the corresponding increase per cell is in the region of 250%. Similar differences are found in the results for liver (Table 18), where there is a consistent fall in the concentration of RNAP in the tissue; in contrast, the amounts per cell remain at the same level over the whole period of development. Although in muscle tissue (Table 19) the figures for concentration and amount per cell show the same general trend, the percentage increases in ASP, LP, and PN are much greater when expressed as amounts per cell. Only in heart tissue, in which the cells show little or no change in composition, are the changes in concentration and in amount per cell materially the same (Table 17).

(b) Rates of Cell proliferation:

Since the DNAP remains constant in amount per cell, the total amounts of DNAP per organ provide a relative measure of cell number throughout embryonic development. As a result, it is possible to follow changes in the rate of cell multiplication in brain, heart and liver, where whole organs were taken for analysis. The daily increments of DNAP in the three organs are obtained from large scale graphs, on which DNAP per organ is plotted against incubation age, and from these are derived the average or mid-increments for the 24 hour intervals covering the 11th. to 20th days. By expressing these mid-increments as percent-

ages of the corresponding amounts of DNAP per organ, (as was proposed and developed by Minot (1908) for weight measurements in studies on the growth-rate of an organism) it is possible to obtain a picture of the varying rates of cell multiplication in each tissue throughout ambryonic development.

The resulting curves are shown in Fig. 17 where it is seen that the rate of cell multiplication is remarkably uniform in the brain tissue of 11 to 20 day embryos. In contrast, both heart and liver cells multiply most rapidly between 10 and 14 days, after which there is a steady decline in multiplication rate until the time of hatching. Figures for the actual amounts of DNAP per organ in Tables 16-18 show that the cell number in heart increases 70 times, in liver 54 times, and in brain only 6 times between the 8th day and hatching time.

(c) Changes in average cell mass.

The changes in average cell mass (i.e. wet weight relative to DNAP) for brain, heart, liver and muscle are compared in Fig. 18. During embryonic growth, brain, liver and muscle cells all increase in mass, while the heart cells show little change in the average weight. The mass of the brain cells increases steadily from 8 to 18 days, and the final values represent an increase of about 200% over the

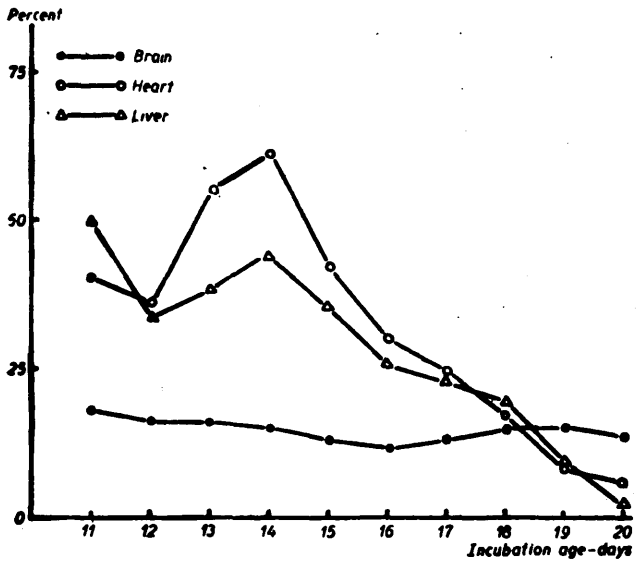


Fig. 17. Percentage increments of deoxyribonucleic acid (DNAP) per organ for 24 hour intervals during embryonic development of chick brain, heart and liver.

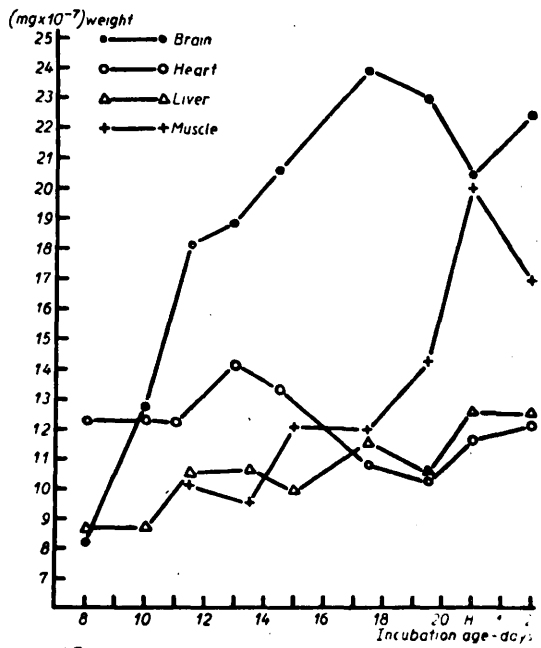


Fig. **18.** Variations in cell mass in chick brain, heart, liver and skeletal muscle during embryonic development. Cell mass calculated from fresh weights of organs on the basis that each chick cell contains 2.35 μg DNAP. *H* on time scale for incubation age refers to hatching time.

mass of 8-day brain cells. Muscle cells increase approximately 100% between the 12th day and hatching time largely owing to an abrupt rise in cell mass after 18 days. The change in liver cells is small and gradual by comparison since they increase only by about 50% between the 8th day and hatching time. Comparison of the relative cell weights in the four tissues of the 2-day old chicks show that heart and liver cells have the same mass, muscle cells are about 35% heavier, and brain cells are 80% heavier (Tables 16-19).

(d) Changes in cell composition:

Fig.19 shows that the acid-soluble phosphorus (ASP) increases in brain and muscle cells in the same way as does cell mass. Again the increase in muscle cells is particularly abrupt in the two days before hatching. Heart and liver cells both show a slight but consistent decline in ASP content, the amounts per cell being highest at the time of rapid cell multiplication in these tissues (Fig.17).

The increase in the phospholipid (LP) content of the brain cells is very large by comparison with the corresponding increases in liver and muscle cells (Fig.20). After hatching, the brain cells contain more than five times more phospholipid than do 8 day cells, while muscle cells increase their content two-

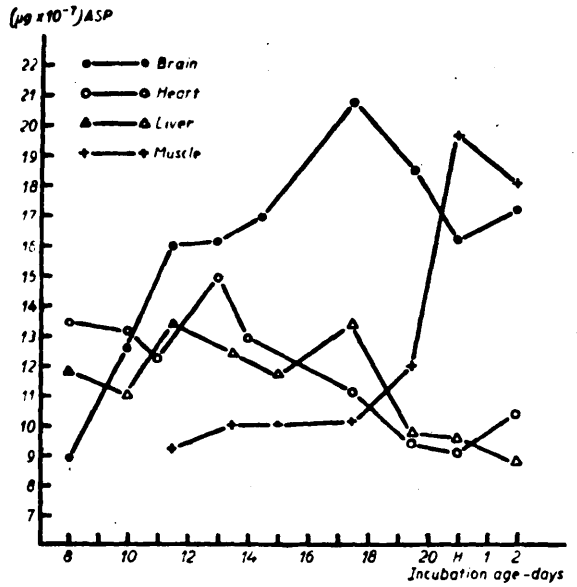


Fig. 19. Variations in amount of acid soluble phosphorus (ASP) per cell in chick brain, heart, liver and skeletal muscle during embryonic development.

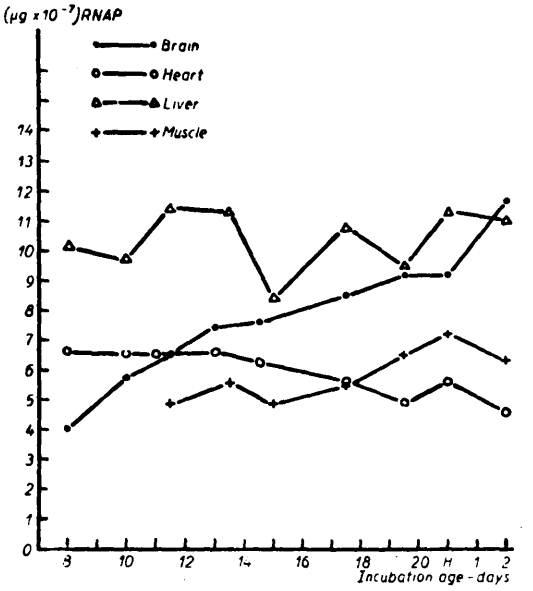


Fig. 21. Variations in amount of ribonucleic acid phosphorus (RNAP) per cell in chick brain, heart, liver and skeletal muscle during embryonic development.

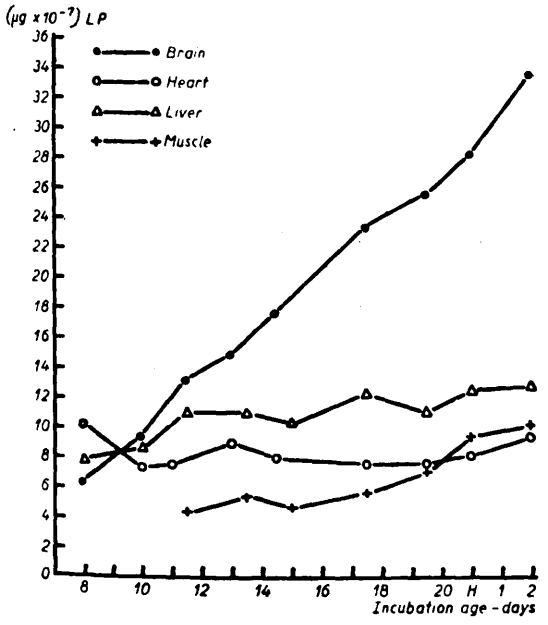


Fig. 20. Variations in amount of lipid phosphorus (LP) per cell in chick brain, heart, liver and skeletal muscle during embryonic development.

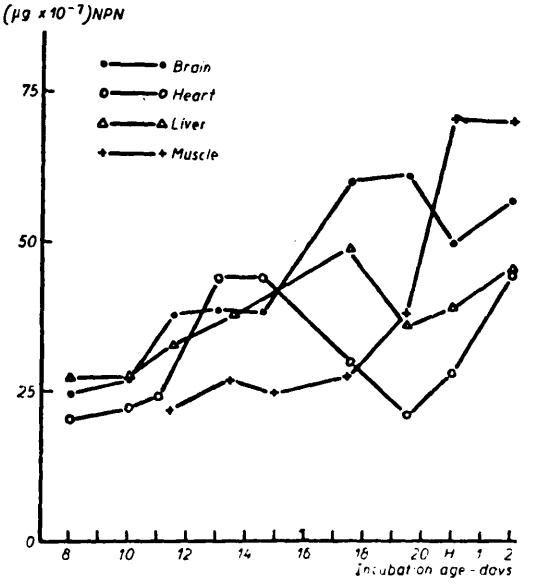


Fig. 22. Variations in amount of non-protein nitrogen (NPN) per cell in chick brain, heart, liver, and skeletal muscle during embryonic development. NPN does not include N present in nucleic acids.

fold after the 12th day. The increase per cell in liver tissue is smaller, and there is no significant change in the phospholipid content of heart cells. The general course of these changes in all four tissues follows closely the pattern found for changes in cell mass (Fig.18).

Changes in the amounts per cell of RNAP are shown in Fig.21. Again there is a steady and comparatively large increase in the RNAP content of brain cells, bringing the amount after hatching to three times that in the 8-day cells. Muscle cells increase their RNAP content by about 50% in the period before hatching, while heart cells lose about 30% of their RNAP in the course of embryonic development. The amount in the liver cells does not change significantly, and is maintained at a relatively high level in comparison with the amounts per cell in the other tissues.

In the non-protein nitrogen (NPN) fraction are included all the nitrogenous compounds present in the acid-soluble and lipid fractions. Muscle tissue shows an abrupt increase per cell of this rather complex mixture of substances over the three days preceding hatching (Fig.22). This rise of just over 100% in the NPN per cell coincides with similar increases in cell mass and ASP content (Figs.18 & 19). The NPN content of brain cells increases from the 8th

day and reaches a steady level on the 17th day, while a rather similar, though smaller, increase occurs in the NPN content of liver cells. The fluctuations in the content of the heart cells are large enough to be considered significant, and there is apparently a temporary fall in their NPN before and during hatching.

Changes in the protein N (PN) content of the cells are shown in Fig.23. The embryonic development of brain, liver and muscle cells is characterised by large increases in the amounts pf PN per cell; in contrast the protein content of heart cells shows no significant change until hatching. The largest rise, over sixfold, occurs in muscle cells, the increase is fivefold in brain cells, and over twofold in liver cells. Such changes should largely account for the increased cell mass found in the same three tissues (Fig.18). From Tables 16-19 it can be seen that the amount of protein per cell in the two day old chick is least in heart tissue, is between 45 and 60% greater in liver and brain, and in muscle cells is twice that in heart cells.

(e) Allometric analysis of results:

In applying the allometric method to the results from brain, heart and liver, the logarithms of the amounts per organ of the various constituents have

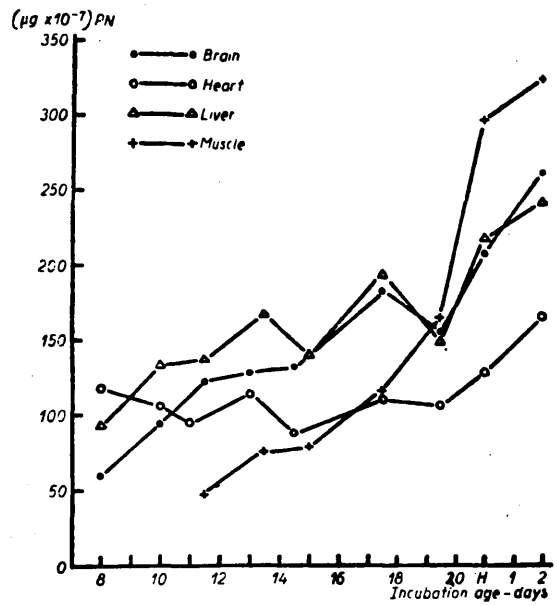


Fig. 23 Variations in amount of protein nitrogen (PN) per cell in chick brain, heart, liver and skeletal muscle during embryonic development. PN does not include N present in nucleic acids.

TABLE **20.**

GROWTH RATES ($k = \tan\alpha$) FOR VARIOUS CELL CONSTITUENTS RELATIVE TO GROWTH RATE OF DEOXYRIBONUCLEIC ACID PHOSPHORUS (DNAP) PER ORGAN

Organ	Incubation period (days)	Weight (mg)	Acid soluble phosphorus (ASP)	Lipid phosphorus (LP)	Ribonucleic acid phosphorus (RNAP)	Protein nitrogen (PN)	Non-protein nitrogen (NPN)
Brain (1)	8–14	3.12	2.07	2.28	1.95	2.04	–
Brain (2)	15–20	1.26	1.28	1.61	1.27	1.31	1.70
Heart	8–20	0.99	0.99	0.99	0.98	1.04	–
Liver (1)	8–14	1.20	1.09	1.26	1.07	1.23	1.15
(2)	15–20	0.95	1.06	1.02	0.94	1.06	—

been plotted on large scale graphs against the logarithms of the corresponding amounts of DNAP, and the lines have been tested for their 'goodness of fit' by the graphical method proposed by Kavanagh and Richards (1945). Figs. 24 and 25 show the contrasting chemical development of the embryonic heart and brain cells. In the heart the growth rate of the components relative to that of the cells remains unchanged for most of the embryonic period between the 8th day and hatching. In brain, where there is a much smaller increase in cell number for the same embryonic period (log DNAP range is half the length of that for the heart), the relative growth rates of the cell constituents are more rapid, and show a very distinct change at a point corresponding to the 14 to 15th day of embryonic development.

The relative growth rates for the three organs are summarised in Table 20. (These rates are represented by $k = \tan$ and in the allometric expression $y = bx^k$). A distinct change in slope occurs in the relative growth rates of components in brain and liver between the 14th and 15th day of development; the only exceptions were the NPN fractions in both tissues and the ASP in liver. The constant values for heart over the whole period are all close to unity as might be expected if the cells are multiplying with little or

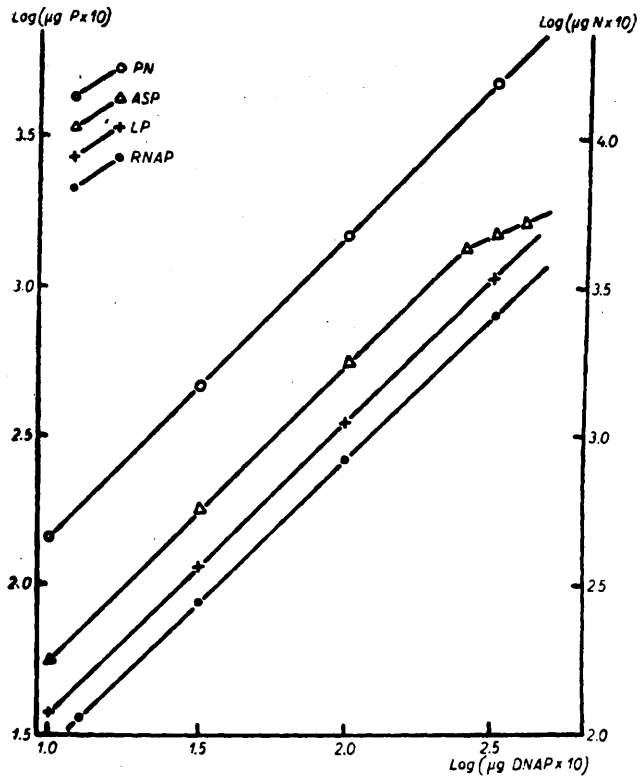


Fig.**24** Growth rates of cell constituents of the chick embryo heart relative to the growth rate of total deoxyribonucleic acid phosphorus (DNAP) per organ. PN-protein nitrogen: ASP- acid soluble phosphorus: LP- lipid phosphorus: RNAP- ribonucleic acid phosphorus.

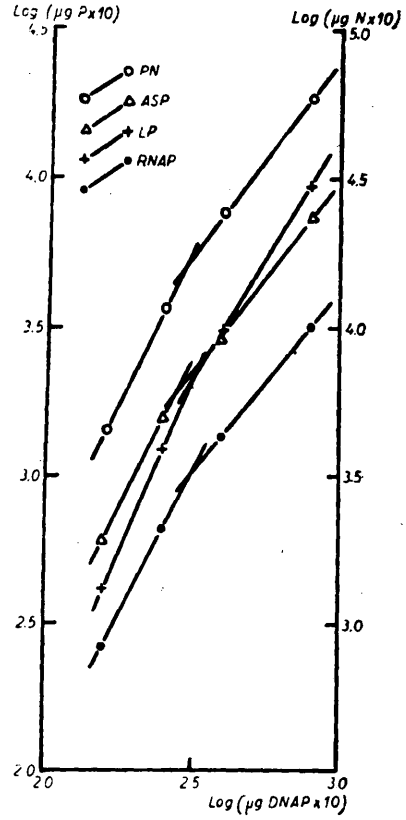


Fig.**25.** Growth rates of cell constituents of the chick embryo brain relative to that of the total deoxyribonucleic acid phosphorus (DNAP) per organ.

no change in the amounts of cellular constituents. Brain is distinguished by the rapid rate of accumulation of cellular material compared with the rate of increase in cell number; in particular, phospholipids are increasing per cell at a faster rate than the other constituents in both phases of development. In liver, the cells increase their PN and LP content (and hence their weight) more rapidly in the first phase of development than in the second, and in this organ the rates of synthesis of these two components appear to be closely related.

When the relationships between the components themselves are examined, a close correspondence is found between the relative growth rates of ASP and RNAP in all three tissues (Table 20). The relative growth rates of RNAP are consistently lower than those of PN, indicating that the PN increases in proportion to the RNAP in the developing cells. There is, on the other hand, some correlation between the values for LP and PN in liver, whereas, in brain, LP accumulates much more rapidly in the cells than does PN. Finally, it is evident from Table 20 that the relative growth rate for cell mass follows to some extent the values for LP and PN in all three tissues.

C. DISCUSSION.

The object of this study has been to obtain information on protein synthesis as it is related, on one hand to the other cell constituents, and, on the other, to the physiological development of individual tissues. As there are so many variables in the developing embryonic system some caution is necessary in the form in which results are expressed. When changes in the cell constituents are given as concentrations per unit weight of wet or dry tissue, the results show how the amount of each constituent varies in relation to the tissue substance as a whole. However they do not necessarily reflect the changes in the composition of the average cell, and they tend to obscure the quantitative relationship between one cell constituent and another.

This emerges clearly from the comparison of the results for the four tissues, which were expressed both as concentrations per 100 mg. wet weight and as amounts per cell on the basis of the constancy of DNA per nucleus (Tables 16-19). Only in the case of heart tissue, in which cell composition shows little change, is there close correspondence between the two sets of results. In the other three tissues when results are expressed in terms of concentrations or as amounts per cell, either the trend or the extent

of the changes is quite different. Interpretations are consequently based on the changes in amount per cell of a constituent since these are independent of other tissue variables; this also applies to the allometric results, which show how the rates of accumulation of cell constituents are quantitatively related to one another, in terms of their common relationship to the DNA of the nucleus.

In embryonic heart and liver the highest average content of ASP per cell occurs when cell multiplication is most rapid, and, as seen in Section III, the same association holds during the growth of chick heart explants in vitro. In brain and muscle, however, the increases in ASP per cell coincide with phases during which the amounts of protein per cell are increasing. These increases are particularly rapid in muscle tissue as it develops its full functional activity in the period immediately before hatching. As the amounts of NPN and PN per cell also rise abruptly at exactly the same stage of development, these results as a whole are in good agreement with the earlier observation (Moog,1947) that a peak in apyrase activity (in units per mg. N) occurred in chick embryo skeletal muscle between 18-21 days. From comparison of the relative growth rates (Table 20), the ASP would seem to be most closely related to the TNAP in all three tissues, and to

show quite distinct deviations from both the NPN and phospholipid fractions. This quantitative link between the ASP fraction and the formation of RNAP was also apparent in the tissue culture studies described above.

While the amount of phospholipid bears a constant relationship to the amount of protein per cell throughout the whole embryonic development of the liver (as reflected in the similarity of their relative growth rates in Table 20), the ratio lipid P: protein N decreases in heart and muscle, and increases in brain. A constant ratio between phospholipid and protein has also been shown to exist in rat liver, during growth and starvation, and irrespective of sex (Kosterlitz, 1947; Campbell & Kosterlitz, 1950). It may be of some interest that the liver plays a special part in other aspects of phospholipid metabolism. In the adult organ, at least, it is the only source of plasma phospholipids (Entenman, Chaikoff & Zilversmit, 1946); and recently, Popjak and Beekmans (1950) have shown that the uptake of deuterium rapidly reaches equilibrium in the phospholipids and glyceride fatty acids in the rabbit liver, but not in the lung and intestine. Phospholipid accumulation in the chick embryo brain has been investigated by Bieth & Mandel (1950), who find that the rising phospholipid content of brain tissue

involves three distinct growth phases, during which cephalin, lecithin, and sphingomyelin respectively accumulate in the cells. They report a considerable increase in phosphagen during the rapid accumulation of sphingomyelin, and this phase seems to coincide with the comparatively high growth rate of phospholipid relative to DNAP, which we find in the 15-20 day embryo (Fig.9,table 6).

Caspersson (1941) and Caspersson & Thorell (1941) in their microspectrophotometric studies have shown that the nucleotide concentration is much higher in the cytoplasm of the early embryonic blood and liver cells than in the corresponding adult cells of the fowl. Their method, employing the ultra-violet absorption of purines and pyrimidines at 260 mμ., determines both the RNA (or polynucleotide) and the individual nucleotides present in the cytoplasm, but does not distinguish between the two. In apparent contrast to their results, the chemical techniques used here show that the average RNAP content of the chick liver cell is as high in the 2 day-old chick as in the 8 day embryo (Table 18). This difference cannot be explained on the grounds that the lower total nucleotide concentration of the adult cell is due to the decreasing concentration of the individual (or acid-soluble) nucleotides, since chemical analyses

of fowl tissues have shown that their concentration is actually lower in the embryonic than in the adult chick tissue (Davidson and Waymouth, 1944). It is more probable that the decrease in total nucleotide concentration is, in part, the result of the nearly threefold increase in the amount of protein per cell in the 2 day-old chick liver as compared with amount per cell in the 8-day embryo liver. Thus the apparently lower concentrations in the adult cell may be due to the diluting effect of increased protein and not to a decrease in the absolute amount of total nucleotide per cell.

The threefold increase in the amount of RNAP per cell, which is found in brain tissue during embryonic development, and which Mandel & Beith (1950) also report, is a particular characteristic of brain tissue. As Hyden (1943) has shown, this persistently high RNA or nucleotide content of nerve cells is related to the intense protein synthesis, which normally accompanies nervous activity. The high values for the RNAP content of heart and muscle cells in each coincide with phases of rapid cell multiplication and protein synthesis.

Apart from a temporary fall in the amounts per cell of PN (and NPN) in brain, liver and heart near the time of hatching, (an event which Moog (1947)

suggested might be the result of anoxaemia) the PN per cell was consistently higher in the tissues at the later stages of development. It seems very likely that this rising protein content of embryonic cells is characteristic of their differentiation towards their final adult form. From the 8th day until hatching the protein per cell increases fourfold in brain, about twofold in liver, and over sixfold in muscle; in contrast, there is no change in heart cells, and it is perhaps relevant that the chick heart has reached its adult morphological form by the 8th day of development. However, there is a slight rise in protein per cell in heart tissue after hatching, and, as the amount per cell at hatching is much lower in the heart than in the other tissues (Tables 16-19), it is possible that further differentiation in this organ is delayed over the embryonic period. The general trend of increasing PN per cell is reflected in the accompanying rise in average cell mass in brain, liver and muscle cells (Fig.18), and is in keeping with increases in cell volume which have been observed during the same phase of chick embryonic development (Illing, 1905).

There is no doubt then, that a permanent increase in protein content occurs in the cells of embryonic tissues while they are growing and assuming their adult functions. The same process is evident during cell

development in the salivary gland of Drosophila, where the proportion of cell protein to nucleic acid is much larger in the fully developed cells than in the young, rapidly multiplying cells. (Caspersson & Schultz, 1939) Thorell (1947), too, has found that differentiation during haemopoiesis involves an increase in cytoplasmic protein at the stage between myeloblast and promyelocyte. On the other hand, in cancerous tissue there is a change in cell type to a less differentiated form, and (Tables 31, 32, Section V) show that this is accompanied by a considerable decrease in the average amount of protein and RNA per cell . Here, again, the high nucleic acid concentrations, which Caspersson and Santesson (1942) find in the active cells of malignant tumours, may not be the result of increased amounts of RNA per cell, but may possible follow from a reduction in the protein content.

As has already been mentioned, the allometric data (Table 20) show a close correlation between the growth ratios of ASP and RNAP in all three tissues, and between LP and PN in liver. When RNAP and PN are compared, it is seen that the latter always has the higher relative growth rate. If this difference is to be reconciled with the generally accepted view that RNA is involved in protein synthesis, it must be assumed that an increasing proportion of the protein

accumulating in the cells is not immediately related to the metabolism of RNA. In such circumstances, the ratio of PN to RNAP should increase during growth, as is indeed found in heart, liver, muscle, and even in brain, where the RNAP itself is increasing significantly in the cells. It is also of interest that a very distinct decrease should occur in the relative growth rates of protein and its associated constituents in liver and brain tissue at a point corresponding to the 15th day of embryonic development. This offers some confirmation of earlier observations that a critical phase in embryonic development is reached at about the 14th to 15th day of incubation (Needham, 1931).

SECTION V.

STUDIES ON NORMAL AND PATHOLOGICAL HUMAN TISSUE

A. INTRODUCTION

The work described in this section originally began as a study of quantitative differences in the content of RNA and DNA in bone marrow of normal and pathological subjects (Davidson, Leslie & White, 1948). In that investigation, attention was given to the changes in concentration of RNA and DNA in human bone marrow during illness and therapy, and the results revealed that the concentrations of both nucleic acids were increased above the normal level in hyperplasia of the marrow (e.g. in leukaemia), and on the appearance of more primitive cell types, (e.g. in pernicious anaemia). During treatment of the pernicious anaemia cases, the concentrations returned towards the normal level associated with healthy subjects.

A new and important aspect of the work developed as a result of the suggestion that a quantitative association existed between the DNA content of the nucleus and the genotype (Section, 1, C). The bone marrow material was in some respects particularly suitable for an investigation into the variations in amount per cell of RNA and DNA present in different samples. Determinations could be made directly on

the cells without previous isolation of the nuclei, and it was possible to obtain numerous small samples in which the nucleated cells could quite readily be enumerated. The main limitation was the wide variation in cellularity and cell type found in such material.

As has been illustrated in the earlier sections, quite a new approach to the understanding of cell physiology became possible once it was established that the average amount of DNA per nucleus showed a remarkable constancy in the different tissues of one animal or species (cf. Davidson & Leslie, 1950 a,b). For example, the ratio RNA:DNA acquired a new significance, since it could be interpreted as a measure of the relative amount of RNA per cell.

This new approach gives a much less ambiguous picture of changes in tissue composition. Generally, it has been the custom to express results as concentrations per unit of wet or dry weight, but such information can be very misleading. Because the concentration of any one constituent is a reflection of the relative amounts of many other constituents, there is no means of deciding how a particular change in concentration has occurred. For example, an increase in the concentration of one substance could follow preferential synthesis, or it could merely be the result of unrelated decreases in other substances.

On the other hand, when results are expressed in relation to units of DNA of the cell, it can be seen at once whether the substance in question increases, decreases or remains constant in amount per cell, thus providing a definite biological basis for the interpretation of changes in tissue composition.

B. RESULTS

THE NORMAL GROUP

(a) Sternal Marrow: The samples were obtained from 22 volunteers, and in a total of 27 observations, the DNAP per marrow cell gave a mean value of 8.69 µg. x 10^{-7}, and the RNAP a mean value of 6.9 µg. x 10^{-7}. The mean ratio of RNAP:DNAP for the normal marrow cells was 0.81. The fairly wide scatter of the results is apparent from the values for the observed range and the standard deviation given in Table 21. This scatter is observed also in the results of the other groups, but only in the normal and leukaemic cell results is there a general conformity to a normal distribution.

(b) Peripheral Blood: Analysis of blood leucocytes of 10 individuals gave mean values for DNAP of 7.04 µg. x 10^{-7} per cell and for RNAP 2.42 µg. x 10^{-7} per cell; the mean ratio RNAP/DNAP was 0.35 (Table 21). The mean leucocyte DNAP is lower than the value for normal, the difference being of borderline signifi-

Table 21

Amounts per cell of RNAP and DNAP in normal human tissue.

Tissue	Measurements	Values for Nucleic acid P in μg. x 10^{-7} RNAP	DNAP	Ratio RNAP/DNAP
NORMAL MARROW		27 observations on 22 individuals	27 observations on 22 individuals	
	Mean	6.90	8.69	0.81
	S.E.	3.18	3.09	0.27
	Observed range	2.1 - 14.4	4.0 - 15.0	0.43 - 1.9
NORMAL BLOOD		17 observations on 16 individuals	11 observations on 10 individuals	7 observations on 6 individuals
	Mean	2.42	7.04	0.35
	S.E.	0.74	2.33	0.16
	Observed range	1.1 - 3.1	4.9 - 12.4	0.15 - 0.55
SPERM		11 observations	11 observations	
	Mean	2.45	3.14	0.78
	S.E.	-	1.35	-
	Observed range	0.78 - 4.8	1.8 - 5.8	-

cance (P=0.05), but the corresponding RNAP values differ very significantly (P=0.001). The blood leucocytes are mature, differentiated cells which no longer undergo mitosis.

(c) Reticulocytes: These cells do not contain DNA, but still retain some of the cytoplasmic ribonucleoprotein of the preceeding nucleated stages of development. This was borne out by the analysis of 5 samples of blood rich in reticulocytes, obtained from 2 patients with haemolytic anaemia; the results indicated between 0.13-0.3 μg. x 10^{-7} of RNAP per reticulocyte.

(d) Spermatozoa: Table 21 also shows the results of the analysis of 11 samples of semen which had been examined by Dr. Wachtel for evidence of male subfertility but without any significant abnormality being found. The mean amount of DNAP per sperm is 3.14 μg. x 10^{-7}, a value which is related to the mean DNAP of the normal mature, non-dividing blood leucocyte in the ratio of 1:2.24. There was rather an irregular scatter in the amount of RNAP per sperm, the range being 0.78-4.8 μg. x 10^{-7}, and the mean 2.45 μg. x 10^{-7}. Cytochemical tests suggest the presence of no more than a trace of RNA in sperm heads, and it is probable that the relatively high figures may be due to contamination of the sperm by adherent fragments of cytoplasm from Sertoli cells. This has also

been suggested by the work of Howard & Pelc (1950) with ^{32}P.

(e) Miscellaneous human tissues: Samples of fresh infant liver and kidney, and of pooled adult prostate tissue were used for the isolation in bulk of nuclei, and a small amount of each tissue was set aside for whole tissue analysis. The results, giving the composition of the three tissues in terms of the amounts per cell, are given in Table 22. In liver tissue the amount of DNAP per nucleus was found for this one sample to be 10 μg. x 10^{-7}, while rather lower values of 8.3 and 8.7 μg. x 10^{-7} were obtained for kidney and prostate respectively. The average amounts of RNAP, LP and PN per cell have been calculated on the basis of these values for DNAP per nucleus, and they show clearly that liver cells have a much higher content of RNAP and LP than kidney and prostate cells. The higher DNAP content of the liver cell nucleus is also in accord with experience in other mammalian species (as discussed in Section I, C).

2. The Leukaemic Group.

(a) Sternal Marrows: Samples were obtained on 33 occasions from 17 patients suffering from various types of leukaemia. The mean amounts per cell of 8.62 μg. x 10^{-7} for DNAP, and 7.52 μg. x 10^{-7} for RNAP (giving a mean ratio RNAP/DNAP of 0.99) were

Table 22.

Amounts per cell of RNAP, LP and PN in human liver, kidney and prostate tissue. These results have been calculated on the basis of the average DNAP content of the isolated nuclei.

Tissue	Amounts of phosphorus as $\mu g. \times 10^{-7}$ cell.			
	DNAP	RNAP	LP	PN
Liver	10.0	43	52	753
Kidney	8.3	11	20	401
Prostate	8.7	9	20	578

not significantly different from the normal values (Table 23).

(b) Peripheral blood: An analysis of blood leucocytes from 15 patients examined on 18 occasions revealed that in leukaemic cases the leucocytes possessed a significantly higher mean RNAP of 3.8 $\mu g. \times 10^{-7}$ per cell than the 2.42 $\mu g. \times 10^{-7}$ found in normal leucocytes. However, the DNAP content of the leucocytes remained the same as in those in normal blood (Table 23).

(c) Variation in the Leukaemias: The leukaemic patients were classified clinically and cytologically by Dr. White into acute, subacute and chronic groups, characterised respectively by excess of primitive blast cells, varying proportions of primitive and older cells, and pronounced excess of the more differentiated cells. Table 24 shows the amounts per cell of RNAP and DNAP for each group. There is an apparent trend towards highest RNAP and ratio values in the acute group, intermediate values in the subacute, and lowest values in the chronic cases. However, an analysis of variance does not confirm the significance of the trend for these observations (see Fisher, 1950).

(d) Effect of Therapy on Leukaemic cells: Where a response to therapy occurs, whatever the therapeutic agent or type of leukaemia, the general result is a decline in proportion of primitive blast cells and a

TABLE 23.

Amount per cell of RNAP and DNAP in human leukaemic cells.

Tissue	Measurements	Values for Nucleic acid P in μg.x 10⁻⁷ RNAP	DNAP	Ratio RNAP/DNAP
LEUKAEMIC MARROW		31 observations on 16 patients	33 observations on 17 patients	33 observations on 16 patients
	Mean	7.52	8.62	0.90
	S.E.	3.54	2.93	0.32
	Observed range	2.6 - 17.4	3.9 - 17.4	0.3 - 1.8
LEUKAEMIC BLOOD		18 observations on 15 patients	18 observations on 15 patients	
	Mean	3.3	6.995	0.56
	S.E.	1.775	1.94	0.22
	Observed range	1.0 - 8.8	3.5 - 11.3	0.13 - 1.0
		t test of Significance for Difference between Means		
Normal and Leukaemic Marrow		Not significant	Not significant	Not significant
Normal Marrow and Normal Blood Leucocytes		Doubtful significance	Highly significant	Highly significant
	P =	0.05	0.001	0.001
	Degrees of freedom =	37	33	33
Leukaemic Marrow and Leukaemic Blood		Just significant	Highly significant	Highly significant
	P =	0.02	0.001	0.001
	Degrees of freedom =	50	50	50
Normal and Leukaemic		Not significant	Significant	Significant
	P =		0.02 - 0.01	0.01
	Degrees of freedom =	28	24	24

TABLE 24.

Nucleic acid phosphorus in the marrow cells of different leukaemic groups

Group	No.of patients	Mean values in µg. x 10^{-7} per cell in Acute, Sub-acute and Chronic Leukaemias. RNAP	DNAP	Ratio RNAP/DNAP
Acute	6	8.78	8.88	0.96
Sub-acute	5	6.12	7.98	0.79
Chronic	22	4.68	8.25	0.60

Table 25.

Aminopterin Therapy in a Patient with Acute Paramyeloblastic Leukaemia.

DATE	THERAPY	MARROW STRUCTURE	NUCLEATED CELLS PER MG. OF ASPIRATED MARROW	AVERAGE NAP PER CELL IN $\mu g.x10^{-7}$		RATIO RNAP/ DNAP
				DNAP	RNAP	
14.6.49	None	Predominance of paramyeloblasts	170,800	8.9	11.6	1.3
20.6.49	3 days after 60 ug of Vit. B.12	Unchanged	104,350	9.0	9.9	1.1
29.6.49	After 9mg.of Aminopterin	Fewer blast cells, more mature cells	100,600	8.3	6.8	0.82
13.7.49	Blood	Mature, actively granulopoietic marrow	245,300	9.4	8.1	0.86
1.9.49	None	Blast cells again predominate	181,700	6.4	7.0	1.1
22.9.49	18mg.Aminopterin from 3 to 11.9.49	Blast cells predominate (Postmortem	Cells not enumerated	-	-	1.8 (Postmortem)

rise in the more mature, differentiated cells in the marrow. Changes in the total cellularity of the aspirated marrow are very variable, however, particularly in the acute forms. No very regular changes are found in the average nucleic acid content of the cells, but increased maturity of the marrow cells is often reflected by a fall in the average RNAP and in the RNAP/DNAP ratio. This is illustrated by the summary in Table 25 of the changes occurring during a temporary remission induced by aminopterin in one case of acute leukaemia.

3. The Anaemia Group.

(a) Pernicious anaemias and other megaloblastic anaemias:

It is in this group that the most marked and consistent deviations from normality are encountered in the amounts of cellular nucleic acids.

The marrow was examined on 28 occasions in 12 cases of untreated or relapsed pernicious anaemia or other megaloblastic anaemias, and the results are shown in Table 26. The mean DNAP per cell was 12.57 μg. x 10^{-7} in untreated cases, and this value was significantly higher ($P < 0.001$) than the 8.69 μg. x 10^{-7} DNAP found in the marrow cells of healthy subjects. The same holds for the value of 13.38 μg. x 10^{-7} RNAP per cell as compared with the mean value of 6.9 μg. x 10^{-7} in normal marrow cells. The ratio RNAP/DNAP of 1.06 for these untreated cases also showed a highly significant

Table 26. *Cases of pernicious anaemia and other megaloblastic anaemias*

NAP in $\mu g. \times 10^{-7}$ per cell

		DNAP	RNAP	Ratio DNAP/RNAP
		28 obs. on 12 cases		28 obs. on 13 cases
Group as a whole	Mean	12·6	10·9	0·87
	S.E.	4·56	5·03	0·27
	Observed range	6·6–22·8	2·3–25·1	0·35–1·5
		12 obs. on 12 cases	11 obs. on 11 cases	12 obs. on 12 cases
Group prior to therapy	Mean	12·57	13·38	1·06
	S.E.	4·17	5·19	0·249
	Observed range	8·1–22·8	7·5–25·1	0·69–1·5
		17 obs. on 8 cases	15 obs. on 8 cases	16 obs. on 9 cases
Group during the course of therapy	Mean	12·63	9·09	0·73
	S.E.	4·36	4·21	0·198
	Observed range	6·6–18·8	2·3–17·6	0·35–1·0

[redacted] t *test of significance between means*

		DNAP	RNAP	Ratio RNAP/DNAP
Megaloblastic series as a whole compared with normal series	*P*	<0·001	<0·001	0·2–0·1
	Degrees of freedom	44 Highly significant	44 Highly significant	46 Not significant
Megaloblastic series before therapy compared with normal	*P*	0·01–0·001	<0·001	0·01–0·001
	Degrees of freedom	28 Highly significant	29 Highly significant	30 Highly significant
Megaloblastic series during therapy compared with normal	*P*	0·01–0·001	0·05–0·02	0·8–0·7
	Degrees of freedom	33 Highly significant	33 Significant	34 Not significant
Megaloblastic series before and during therapy compared	*P*	0·7–0·6	0·05–0·02	<0·001
	Degrees of freedom	27 Not significant	24 Significant	26 Highly significant

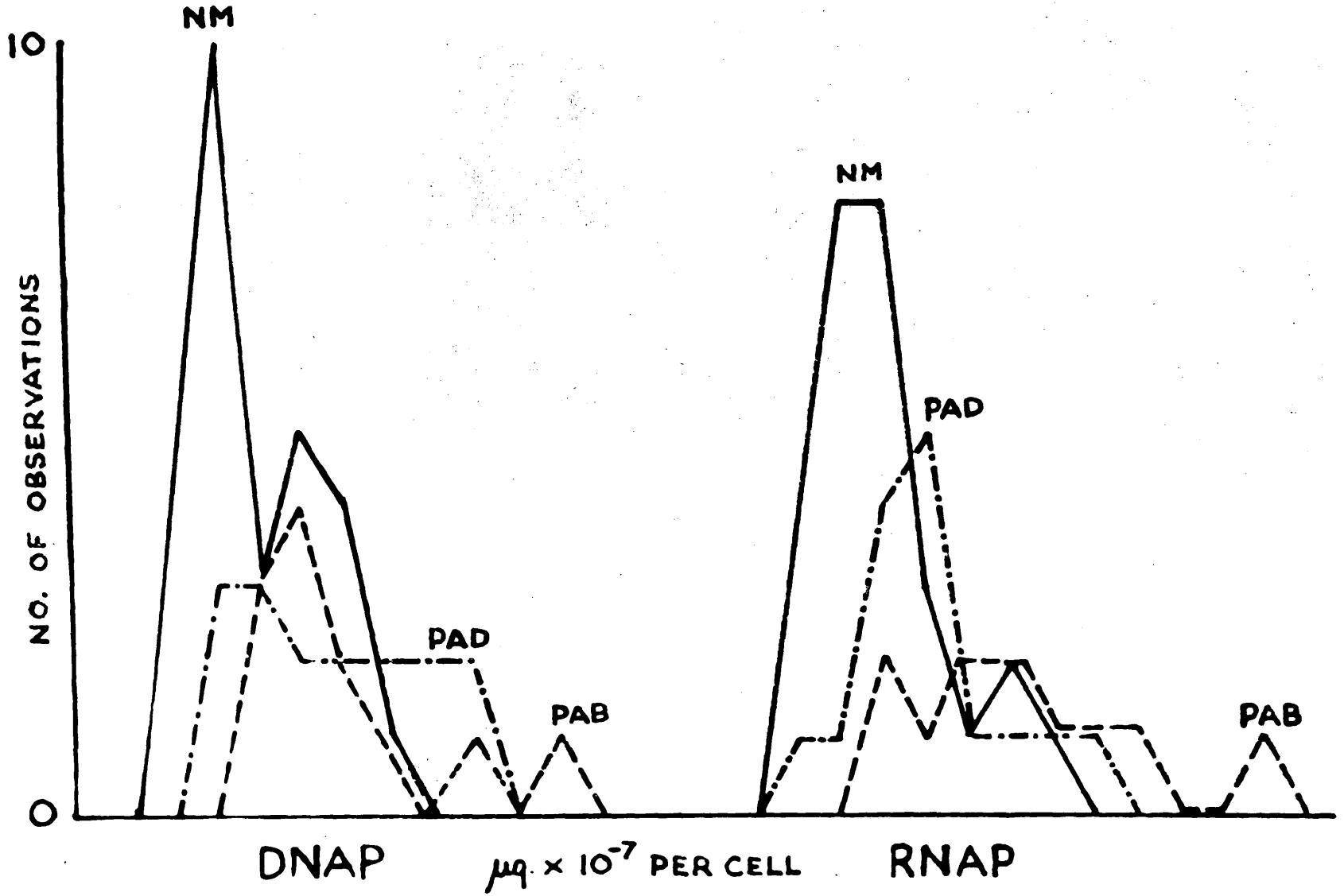
Fig. 26.

Distribution of average Nucleic Acid Phosphorus per marrow cell in Pernicious Anaemia, compared with normal marrow.

NM, 27 observations on normal marrow cells.

PAB, 12 samples of marrow in untreated pernicious anaemia.

PAD, 17 samples of marrow from 8 patients with pernicious anaemia under treatment.


NM
10
NO. OF OBSERVATIONS
PAD
PAB
NM
PAD
PAB
0
DNAP
μg. × 10⁻⁷ PER CELL
RNAP

increase over the normal value of 0.81. Although these means differ so significantly from normal, the individual values were often within the normal range, the distribution curves overlapping and extending to the right of the normal (Fig.26).

(b) Effect of therapy: From the observations made on 9 patients undergoing therapy, it is clear that the mean DNAP per cell did not change significantly during the haemopoietic response (Table 26). There was, however, a return of the marrow to normoblastic erythropoiesis and an improvement in the peripheral blood picture; this was accompanied by a rapid decline in the RNAP per cell towards normal, although the mean value remained significantly elevated ($P = 0.05$-0.02). The ratio RNAP:DNAP also returned to the normal level (Table 26) in the course of treatment. But, from the clinical aspect, Dr. White has emphasised that this group under therapy cannot be considered as returned to normal, either as regards blood picture, marrow cytology or adequacy of therapy.

4. Influence of technique on results.

The marrow aspirated from the sternum consists of cellular fragments suspended in blood. Histological sections prepared from this material show that the fragments represent the true stromal, haemopoietic tissue, whilst the adherent blood contains a mixture

of mature peripheral leucocytes and more primitive cells liberated from the fragments.

In the earlier work (Davidson, Leslie & White, 1948) only the marrow fragments were analysed. The ratio RNAP/DNAP ranged from 3.8 to 1.0 in the normal series, with a value of 2.0 from the means of RNAP and DNAP. In the present series, the whole aspirate was used, and the results express average amounts of nucleic acid phosphorus for mixed populations of primitive and mature cells. This is responsible for the lower mean ratio RNAP/DNAP in the present normal series - 0.81 with a range of 1.9 to 0.43.

Using sternal marrow aspirated from 4 normal individuals, an attempt was made to demonstrate this difference between the composition of whole aspirate and the cellular fragments. The fresh samples were divided at once into 3 fractions, which were analysed separately: (a) whole aspirate, (b) the fragments of marrow remaining after removal with a pipette of the supernatant blood, this last being fraction (c), blood containing mature leucocytes and free marrow cells. The results are given in Table 27.

The RNAP/DNAP ratios in the whole aspirate are much lower than the corresponding ratios for the fragments alone, and 3 of these are similar to the ratios previously found for fragments, but one is

TABLE 27.

Nucleic Acid Phosphorus in aspirated marrow from 4 normal individuals. Each sample was divided into 3 fractions, which were analysed separately:-

(a) whole aspirate, (b) cellular marrow fragments only, and (c) the blood separated by pipetting from the fragments.

NAP expressed as µg. per 100 mg. in each fraction.

		Normal Marrow Sample			
		1	2	3	4
(a) Whole aspirate	DNAP	1.9	3.6	4.9	11.1
	RNAP	1.1	1.6	3.5	10.9
	Ratio RNAP/DNAP	0.58	0.43	0.71	0.98
(b) Marrow fragments	DNAP	3.3	5.8	5.9	25.0
	RNAP	13.6	11.8	16.3	16.7
	Ratio RNAP/DNAP	4.2	2.0	2.75	0.67
(c) Blood separated from the fragments	DNAP	2.5	4.0	4.8	6.7
	RNAP	3.2	2.9	3.5	5.5
	Ratio RNAP/DNAP	1.3	0.73	0.71	0.81

lower. An analysis of variance shows that the higher ratios for fragments are determined by their significantly higher content of RNAP, as compared weight for weight with the whole aspirate ($P < 0.001$).

(a) Effect of suspending marrow samples in saline: An attempt was made to determine whether the suspension of marrow samples in heparinized 0.85% sodium chloride solution, as used in the later technique, resulted in any loss of nucleic acids. Marrow samples from 9 normal individuals and 5 leukaemic patients were each divided half being transferred directly to 10% TCA, and half to 0.5 ml. of 0.85% sodium chloride solution in the usual way. The nucleic acid concentration of the paired samples were then compared on the basis of DNAP and RNAP per 100 mg. of aspirate. Table 28 shows the means of the amounts in the saline-treated samples as compared with the means of the amounts in the samples taken direct into trichloracetic acid.

There is an apparent loss of 10-15% in the RNAP of saline-treated samples, and this is reflected in the lower ratio RNAP/DNAP found in both groups. However, the application of an analysis of variance to these figures, shows that the differences cannot be considered as statistically significant.

5. Analysis of Biopsy Specimens of Human Liver:

The material for this investigation consisted

Table 28.

Effect of suspension of marrow cells in 0.85% sodium chloride solution, prior to trichloracetic acid extraction.

Samples of aspirate have been divided into two portions,
(a) for immediate trichloracetic acid extraction,
(b) for preliminary suspension in 0.85% sodium chloride solution, followed by extraction.
The mean nucleic acid phosphorus content of each group is expressed in ug per 100 mg. of aspirate, and the percentage differences expressed.

		Mean Nucleic Acid Phosphorus in µg per 100 mg. of aspirate		
		Mean DNAP	Mean RNAP	Ratio RNAP/DNAP
9 Normal Samples	(a) Aspirate immediately extracted with trichloracetic acid.	5.75	4.71	1.04
	(b) Aspirate suspended in 0.85% sodium chloride solution	5.82	4.21	0.77
	Percentage Difference between (a) and (b)	1.2	-10.6	
5	(a) Aspirate immediately extracted with trichloracetic acid.	21.34	19.44	0.99
	(b) Aspirate suspended in 0.85% sodium chloride solution	22.42	16.64	0.79
	Percentage Difference between (a) and (b)	5.1	- 14.2	

of needle-biopsy specimens or normal and pathological liver supplied by Dr. S. Sherlock and Dr. J.C. White, Postgraduate Medical School, Hammersmith, London. In contrast to the work which has already been described in this section, this study of liver tissue could not be used to give direct information on the amounts of RNAP, DNAP and RN per cell, since it was impossible to count cell number or to isolate nuclei in such samples. But, if the assumption is made that the average amount of DNAP per nucleus is constant (and on the basis of the information reviewed in Section I, C, this is not unreasonable), then it is possible to calculate the amounts of RNAP and RN per cell from their ratio to DNAP. Results expressed in this way are compared with those obtained by the conventional method of assessing the amounts per unit weight of tissue. (Table 29).

Specimens of 13 normal livers had the highest average concentration of RN (2429 µg.N/100 mg.) and the highest average values of RNAP and RN per cell at 27.2 µg. x 10^{-7} and 1250 µg x 10^{-7} respectively. In obstructive jaundice the greatest change from the normal was a reduction in the RN concentration, but it does not seem sufficient to alter significantly the amount of PN per cell, which like the RNAP, is only slightly less than normal. Cirrhosis of the liver

TABLE 50.

Content of RNAP, DNAP and RN in Needle-Biopsy Specimens of Liver. Average amounts of RNAP and RN per cell calculated on basis of average DNAP per cell of 10.0 µg. x 10^{-7}.

Specimen	No. of Observations	µg./100 mg. of fresh liver			µg. x 10^{-7} per cell		RATIO
		RNAP	DNAP	RN	RNAP	RN	RN/RNAP
NORMAL LIVER	9-13	52 (36-74)*	21 (10-36)	2429 (938-5920)	27.2 (17-47)	1250 (680-2520)	47 (26-94)
OBSTRUCTIVE JAUNDICE	7	48 (12-66)	19 (4-30)	1648 (675-2330)	25.6 (22-32)	1180 (470-3200)	44 (21-99)
CIRRHOSIS	5	55 (32-156)	22 (19-46)	1827 (1356-2200)	23.8 (13.6-34)	850 (730-1050)	38 (34-42)
INFECTIVE HEPATITIS duration 3-16 days	6	60 (25-96)	32 (25-44)	2220 (1064-4700)	17.9 (10-21.8)	600 (320-1060)	40 (31-63)
INFECTIVE HEPATITIS duration 25-35 days	3	52 (27-57)	22 (13-27)	1927 (1594-2160)	25 (21.4-31.7)	980 (590-1520)	38 (28-48)

* Ranges given in brackets

produced even smaller changes in concentration from the normal; the most noticeable change in this disease was the 32% reduction in the amount of RN per cell as compared with the normal value.

The most dramatic changes in liver composition were those produced by infective hepatitis. Although the concentration of RN was in the normal range, there were distinct increases above the normal in both RNAP and DNAP concentrations. The extent of the changes become fully apparent when the results are expressed as amounts per cell when it is seen that the average RNAP per cell is reduced by 34% and the RN by nearly 50% in the first 3-16 days of the disease. In infective hepatitis of 25-35 days duration, there is an indication of a return to normal in both in concentrations and in amounts per cell, although this observation must be qualified by the fact that only a small number (3) of samples were analysed.

In contrast to the distinct variations between the amounts of RN and RNAP in normal liver and the amounts in the pathological liver tissue, the ratio of RN/RNAP shows no very definite trend, and lies within the narrow limits of 38-47. If any change does occur, it would appear that liver damage causes a reduction in the normal ratio.

C. DISCUSSION

Until recently the biochemist and cytologist have described tissue composition in quite different terms. Since microchemical analysis has not yet sufficiently advanced to enable measurements on individual cells to be made, the biochemists has generally had to study tissue constituents in terms of their concentrations per unit weight of the fresh or dried material. On the other hand, the cytologist using the method of visual microscopy has been in a position only to give a qualitative account of the chemical nature of different cell types. The procedure of expressing analytical results in terms of amounts per unit of DNA or DNAP, (and of so obtaining information on the average composition per cell) allows the direct comparison of chemical estimations with the results of microscopical examination, and to some extent bridges the gap between the two fields of study.

This method of determining cell composition depends, of course, on the degree of constancy of DNA per nucleus in the somatic cells. From the earlier discussion, it is clear that there are two main sources of departure from the constant amount of DNA associated with diploid cells. (Section I,C). First, the amounts of DNA undoubtedly increase in the nucleus at some stage in the life cycle of

dividing cells, and secondly, the DNA is increased in polyploidy in a step-like manner in keeping with the multiple increase in chromosome number.

The investigation on bone marrow has helped to extend our information on the first point. In both the normal and leukaemic marrows (Tables 21, 23) the average amount of DNAP per cell is 19% higher than that of the DNAP of the corresponding blood leucocytes, though the difference in each case is only of borderline significance. Although marrow is a very heterogeneous tissue, and the number of cells actually undergoing division is never high, the number preparing for mitosis is probably far greater. It is likely, then, that the increased DNA per nucleus over that of the blood leucocytes is a reflection of the greater rate of proliferation in the more primitive marrow cells. The lower value of DNAP per nucleus in the normal blood leucocytes is also closely similar to the figure (DNA = 6.85 μg. x 10^{-6}) given by Mandel, Metais and Cuny (1950), who find little difference in the amount amongst different mammalian species.

There is, on the other hand, good agreement between the bone marrow value of 8.69 μg. x 10^{-7} DNAP per nucleus and the single observations on human kidney and prostate cells giving values of 8.3 and 8.7 respectively. The still higher mean value of 10.0 μg. x 10^{-7} DNAP per nucleus in human liver is more readily understood, since

it is in accord with the comparable high values in bovine and rodent liver tissue, and is almost certainly the result of polyploidy, which Leuchtenberger, Vendrely & Vendrely (1951) showed to be responsible for the high average value in rat liver.

The mean content of human sperm of 3.14 μg. x 10^{-7} DNAP per cell is rather lower than might be expected on the assumption that the mean values found for normal marrow, kidney and prostate cells represent the normal diploid amount. However, when the sperm value is compared with the DNAP of normal blood leucocytes (Table 21), there is rather closer agreement to the expected 2:1 ratio for the DNAP in diploid and haploid cells. This again suggests that the average DNAP content of the marrow cells is elevated as the result of their relatively high rate of division. Tissues proliferating even at a very slow rate will exhibit this effect, which may help to explain the different degrees of departure from the 2:1 ratio which might be expected between the DNAP content of individual somatic cells and the corresponding spermatozoa.

The values for the average DNAP per cell for normal and leukaemic marrows are very similar, as are the means for the cells in normal and leukaemia blood (Tables 21,23). Metais and Mandel (1950) who have studied the DNAP of human blood leucocytes with

chemical techniques similar to those employed here, have obtained results similar to those of the present author, both in the values of the means, and in the lack of any significant difference between normal (6.921 μg. x 10^{-6} DNA) and leukaemic (7.011 μg. x 10^{-7} DNA) leucocytes; the observed ranges and standard errors are, however, considerably smaller than those in the present study. Davison & Osgood (1951) have also studied the DNAP of normal and leukaemic human leucocytes, and have observed ranges similar to those encountered here, though with rather lower means. For human granulocytes and human lymphocytes (two quite different cell types obtained from leukaemic blood) they obtained mean values of 6.25 μg. x 10^{-6} and 5.84 μg. x 10^{-6} respectively for the DNA content of the nucleus.

It is only in the marrow cells of cases of pernicious anaemia that a striking change is found in the average DNAP content per cell. This reaches a value 45% higher that the average for normal marrow, 79% higher than in normal blood leucocytes, and almost exactly four times the mean amount found in sperm. Although hyperplasia of the marrow occurs in some cases of megaloblastic anaemia, it seems unlikely that this great elevation in DNAP content is entirely associated with rapid proliferation of the immature

cells. On the contrary, the extent of the increase rather suggests that cell division leading to maturation is blocked in pernicious anaemia at a stage where the DNAP per nucleus is close to twice its normal diploid amount.

Since the RNA content of the cell is associated primarily with the intensity of protein synthesis, it is subject to greater variation than the DNA. The lowest values of RNAP per cell are found in blood leucocytes, which according to the cytochemical evidence lose their cytoplasmic and nucleolar RNA during maturation. In leukaemic patients, the blood leucocytes have an RNAP content which is considerably lower (50%) than that of the leukaemic marrow cells, but is still higher (57%) than the RNAP of normal leucocytes. However, the general tendency for a fall in RNAP to occur with increasing cell maturity is in line with the concepts developed by Thorell (1947) in spectrophotometric studies on haemopoietic cells.

There is no significant difference between the RNAP per cell in normal and leukaemic marrow, but in pernicious anaemia, the marrow cell has an RNAP content which is 94% higher than the normal. During the course of therapy this declines to an average which is only 32% above the normal, but is still

significantly high. This recalls the situation, in leukaemia during treatment, where a fall in RNAP per cell frequently accompanies the increasing maturity of the marrow cells. High RNAP content of marrow cells is, therefore, associated with the more intensive mitotic activity of the primitive cell types in both normal and pathological conditions.

For the entire series of bone marrow and blood cells, the ratio RNAP:DNAP remains within the range 0.35-1.06, and, as might be expected, the only other tissues, in which this ratio covers the same range, are thymus and spleen. In pernicious anaemia, where the DNAP per cell is so greatly elevated, the ratio (1.06) lies at the upper end of the scale. But even with this abnormally high DNAP content, the RNAP:DNAP ratio consistently follows the trend of the RNAP per cell in decreasing when cell maturation occurs, whether this is exemplified by the difference between marrow cells and blood leucocytes, or by the influence of therapy on marrow cells in leukaemia and pernicious anaemia.

No direct determination of DNAP per nucleus was possible in the case of the liver biopsy specimens, but the remarkable constancy in the average DNA per nucleus in normal liver, in livers from fasted animals, and in liver tumours (Section I,C) strengthens the

assumption that the average DNAP per nucleus in the present specimens is approximately 10 μg. x 10^{-7}.

It is only when the residual nitrogen (RN) is expressed in terms of its amount per cell that the striking changes in cell composition during liver disease become fully apparent. In infective hepatitis, particularly, the decrease to half the normal RN content of the cell is not paralleled by a change in the RN concentration, which remains nearly as high as in normal liver. At the same time, the concentrations of RNAP and DNAP are deceptively large, since the amount of RNAP in the cell is lowered approximately in proportion to the decrease in RN, the ratio RN/RNAP being only slightly less than the ratio in normal liver.

The outstanding feature in these results is the drop in RNAP and protein (or RN) content per cell which follows closely the degree of liver damage. It is of some interest to examine other instances of this pattern of change in liver cells, without necessarily suggesting that there is any more than a superficial resemblance in cell composition.

Mandel, Jacob and Mandel (1950) have studied the effect of prolonged protein deficient diet on rat liver, kidney and brain, and their results are shown in Table 30 in which the amounts per cell calculated on the basis of the reported concentrations are also shown. As in hepat-

TABLE 30.

Changes in concentrations and cellular composition of RNAP, DNAP and PN in rat liver, kidney and brain during prolonged protein deficiency. Amount per cell based on ratio to DNAP.

TISSUE	Initial Wt. of Rat	µg. per 100 mg.			Relative amount per cell	
		RNAP	DNAP	PN	RNAP	PN
LIVER						
Controls	170	102	19.8	2820	5.14	142
Deficient	160	112	36.8	2420	3.01	66
Controls	235	96	17.1	3120	5.60	182
Deficient	237	77	26.3	2180	2.95	83
KIDNEY						
Control	189	65	27	2400	2.4	89
Deficient	196	53	43.5	2160	1.2	50
Control	234	59	30	2280	1.9	76
Deficient	236	49	40	2000	1.2	50
BRAIN						
Control	254	42	12.8	1540	3.3	120
Deficient	254	41	13.4	1450	3.1	108
Control	289	39	10.4	1330	3.8	128
Deficient	285	38	11.2	1450	3.4	129

Table based on results of Mandel, Jacob and Mandel (1950)

itis, the liver cells lose their protein and RNAP to an extent which cannot be appreciated when changes in concentration alone are reported. Similar alterations are produced in kidney cells, but not in brain cells. The results for liver tissue from rats on a protein deficient diet are supported by Muntwyler, Seifter and Harkness (1950) who show that the reduction per cell of both RNA and protein is borne mainly by the small granule fraction.

A series of investigations has been in progress in recent years on the changes produced in rat liver during the induction of hepatomas with various carcinogenic agents. Where they have included determinations of DNA, it is possible to see how changes based on concentrations differ from those expressed as amounts per cell. When, for example the carcinogenic dye, 3'-methyl-4-dimethylaminoazobenzene (3'Me-DAB) is fed to rats over a number of weeks, Griffin, Nye, Noda & Luck (1949) found that the nitrogen and RNA concentrations fell but slightly during hepatoma development, while the DNA concentration increased steadily.

When the amount of nitrogen and RNA are expressed as ratios to DNA so as to indicate the average content per cell, the picture is very different. Table 31 shows that during carcinogenesis a steady but very pronounced fall in the protein content of the cells

TABLE 31.

EFFECT OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE ON THE COMPOSITION OF RAT LIVERS*

	CONTROL BASAL DIET	WEEKS FOR WHICH DIET CONTAINING DYE WAS FED				HEPATOMAS
		2	4	6	8	
	mg. per milligram DNA					
Nitrogen	9.48	6.24	5.61	5.10	4.88	2.70
Nonprotein nitrogen	0.68	0.46	0.48	0.42	0.46	
Phosphorus	1.05	0.80	0.72	0.63	0.65	0.36
Nonprotein phosphorus	0.30	0.25	0.24	0.22	0.21	
Ribonucleoprotein as ribose	0.56	0.31	0.33	0.29	0.25	0.18
Riboflavin (μg.)	9.3	5.0	4.0	2.8	2.5	

* Calculated from data of Griffin, Nye, Noda, and Luck (1949).

TABLE 32.

EFFECT OF 2-ACETYLAMINOFLUORENE ON THE COMPOSITION OF RAT LIVERS*

	CONTROL BASAL DIET	WEEKS FOR WHICH DIET CONTAINING CARCINOGEN WAS FED						24 WEEKS +4-WEEK BASAL DIET	
		2	4	6	14	20	24	Liver	Tumor
	mg. per milligram DNA								
Nitrogen	9.3	9.9	8.0	7.3	9.7	11.8	10.1	8.4	6.2
Phosphorus	1.1	1.3	0.9	0.9	1.1	1.4	1.3	1.1	0.7
RNA as ribose	0.51	0.54	0.32	0.37	0.41	0.60	0.44	0.63	0.38
Riboflavin (μg.)	9.3		6.6		6.8		7.5	5.6	1.0

* Calculated from data of Griffin, Cook, and Cunningham (1949)

TABLE 33.

EFFECT OF AMINOAZO DYES ON THE COMPOSITION OF RAT LIVER FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF HOMOGENATES*

	CONTROL WITHOUT DYE	AB	4′-Me-DAB	DAB	3′-Me-DAB
			mg. per milligram DNA		
Protein in:					
whole liver homogenate	65.5	66.5	61.0	48.6	25.65
nuclear fraction	8.3	6.67	8.1	7.75	8.1
large cytoplasmic granules	21.5	21.4	19.4	12.54	3.88
small cytoplasmic granules	9.1	10.19	9.45	6.34	2.88
supernatant fluid	25.3	25.20	23.5	19.6	10.00
Ribonucleic acid in:					
whole liver homogenate	2.93	2.67	2.31	2.15	1.12
nuclear fraction	0.24	0.23	0.19	0.16	0.29
large cytoplasmic granules	0.95	0.84	0.74	0.54	0.17
small cytoplasmic granules	0.96	0.86	0.71	0.46	0.23
supernatant fluid	0.66	0.69	0.58	0.52	0.36
Riboflavin (μg.) whole homogenate	5.3	5.3	6.1	3.1	1.4
nuclear fraction	0.32	0.43	0.60	0.32	0.42

AB = 4-aminoazobenzene (non-carcinogenic)
4′-Me-DAB = 4′-methyl-4-dimethylaminoazobenzene (weakly carcinogenic)
DAB = 4-dimethylaminoazobenzene (moderately strongly carcinogenic)
3′-Me-DAB = 3′-methyl-4-dimethylaminoazobenzene (strongly carcinogenic)

* Calculated from the mean values of the figures given by Price, Miller, Miller, and Weber (1949)

occurs, accompanied by a corresponding but less marked decrease in RNA. Another carcinogen, 2-acetyl-aminofluorenem shows a somewhat different mode of action during the induction period, but produces tumour tissue with the same cellular characteristics, as can be seem in Table 32 in which the results have been recalculated by the present author from the data of Griffin, Cook & Cunningham (1949).

Additional information about the changes occurring in the intracellular distribution of protein, RNA and riboflavin during the induction of liver tumours, can be obtained from the data of Price, Miller, Miller & Weber (1949). When their results are recalculated in terms of the ratios of these constituents to DNA (Table 32), it becomes apparent that the nuclear fraction undergoes little change in composition under the influence of the most active carcinogen, 3'-Me-DAB; on the other hand, the proportions of protein, RNA and riboflavin are seen to be considerably reduced in the large and small granule fractions.

In an earlier section (I,B) the intracellular distribution of enzymes in mouse hepatoma 98/15 has been compared with the normal distribution found in the C3H mouse liver. When activities are expressed as units per unit of DNA (Table 33), it becomes clear that the principal change in tumour tissue is a marked reduction in activities per cell of certain enzymes. In Table 33 it is seen that

TABLE 33.

Enzyme activities in cells of normal and tumour tissues expressed as units of activity per unit of DNA.

Tissue	Enzyme Content	Nuclear Fraction	Large Granules	Small Granules	Cell Fluid	Homogenate	Authors REF. NO.
C3H Mouse liver	Adenosine-triphosphatase	22	35.4	10.7	3.6	70.5	191
Hepatoma 98/15	" "	13.6	4.7	12.7	5.0	36.1	191
C3H Mouse liver	Succin-oxidase	37.5	107	8.2		190	189
Hepatoma 98/15	" "	5.1	17.6	2.3		30	189
C3H Mouse liver	Cytochrome oxidase	60.5	240	13	0	306	189
Hepatoma 98/15	" "	7.7	38.1	9.8	0	60	189
C3H Mouse liver	DPN-cyto-chrome c reductase	2.8	8.8	18.4	1.1	31	102
Hepatoma	" "	5.1	9.5	18.0	2.7	35	102

Actual ratio has been multiplied by 100 to make comparison with other results somewhat easier.

the activities of adenosinetriphosphatase, succinoxidase and cytochrome oxidase are all much lower per cell in tumour tissue homogenates than in normal liver; the major part of this reduction is, in each case, borne by the large granule fraction. Only the adenosinetriphosphatase shows an increased activity in one of the cell fractions (the small granules) in the tumour tissue. In complete contrast, the activity of DPN cytochrome c reductase increases or remains the same as the normal in the various fractions of tumour tissue.

From the extent of the decrease in protein content in tumour cells as compared with the normal (Tables 30,31), it would appear that the activities of many enzymes must be reduced in the tumour cells. Although this reduction of protein recalls the general pattern of change found in liver cells damaged by disease, starvation or malnutrition, it is clearly important to have much more information about the accompanying changes in enzyme activity and distribution within the cells.

SECTION VI

GENERAL SUMMARY AND CONCLUSIONS

An important difference in the behaviour and function of the nucleic acids, RNA and DNA, has become apparent in recent years. The RNA is distributed widely throughout the cell, in nucleus and cytoplasm, and is known to vary considerably in amount according to the state of activity of the tissue. For example, increased concentrations of RNA in cells and tissues have for some time been associated with protein synthesis and cell proliferation (Section 1,A). More recent work has shown that in embryonic, regenerating and pathological tissue, the amounts of RNA in the whole cell and in its nuclear and cytoplasmic regions differ greatly from the normal adult levels (Section I,B).

The DNA, on the other hand, is confined to the nucleus, where it forms part of the chromosome structure, and shows a remarkable degree of constancy in amount per nucleus within the somatic cells on one animal or species. In large populations of cells in normal tissues, the constant amount of DNA per nucleus is on the average little altered by circumstances which produce large changes in the RNA and protein content of the cells (Section I,C).

This constancy of DNA in the cell nucleus has

opened the way for the biochemist to study living tissues in terms of their cell content and cell composition. Once the average amount of DNA per nucleus is known for the cells of a particular animal, it is an easy matter to follow changes in cell number from determinations of total DNA content. In the same way, average cell mass and cell composition can be determined if values for tissue weight and chemical content are expressed in terms of units of DNA (Section II,D). Although the microchemist cannot yet compete with the cytologist in his ability to study the individual cell, he can, at least, give a quantitative account of the changes in average cell number and composition occurring in large populations of cells.

This new approach to the study of living tissue has been developed in the course of the work described above, where it has been applied to the study of embryonic and tissue culture growth (Sections III & IV). The development of pathological conditions in human tissue has also been investigated by the same technique (Section V). In the course of the work it was found possible to compare results expressed in terms of cell composition with those expressed in the conventional manner, as concentrations per unit weight of tissue (Sections IV & V). This has shown that such concentrations can be entirely misleading, since the tissue weight itself represents a changing complex of variables.

Changes in concentration are, in many instances, very different from the accompanying changes in cell composition. In chick embryo brain, for example, the RNAP concentration decreases with age, and the interpretation which appears most likely is that RNAP is a less active component in the more highly differentiated brain tissue (Section IV). When, however, the RNAP content per cell is found to rise considerably during development, the conclusion to be drawn is that RNA becomes of increasing importance to the nerve cells as they approach their functional stage (cf. Hyden, 1943). Nor can such errors in interpretation be avoided by expressing results as concentrations per unit weight of nitrogen; in the chick liver, for example, the RNAP content per cell is found to remain at the same level throughout development, but its concentration decreases largely as a result of the increasing protein content of the cell.

These discrepancies (and others may be found in the general literature, cf. Davidson & Leslie, 1950,b) emphasise the importance of describing tissue growth and composition in biological terms. This can only be done adequately by following events during growth and development in three dimensions. First, it is necessary to establish cell number; secondly, to measure the changes in average cell mass; and finally, into this framework

can be fitted the information on the amounts per cell of the constituents under investigation. This procedure eliminates the confusing picture obtained by measuring changes in concentration. Cell composition is seen to vary in relation to a single chemical constituent, which increases in direct proportion to the total number of cells.

This method has revealed that the growth of chick heart explants and of chick embryo tissues is in each case characterised by a particular pattern of chemical development (Section III;IV). In explants, cultivated in vitro, normal differentiation of the cells does not occur, although it is generally accepted that a process of 'dedifferentiation' to a more primitive cell type follows the isolation of the tissue from the animal body (cf. Willmer, 1945). This view is, however, not supported by the present work.

The chemical changes during 6 days of growth in these explants are characterized by an initial two to threefold increase in the amount per cell of protein and phospholipid. When cell proliferation is most rapid in its logarithmic phase between 48 and 144 hours, the constituents decrease in amount per cell until they reach their original levels present in the freshly cut explants. On the other hand, the cellular contents of both acid-soluble phosphorus and RNAP rise to a peak in this phase of rapid cell proliferation, only to return again to the initial values when

cell multiplication is much slower after 144 hours. Because the synthesis of DNA is delayed in the first 48 hours of growth, it is concluded that protein and phospholipid components of the cells are synthesized before the DNA of the chromosomes.

This pattern of change is almost certainly related to the chemical processes associated with a phase of intensive cell multiplication, and not to a process of 'dedifferentiation', which, as other investigations suggest (Sections IV & V), would be accompanied by a reduction in the protein content of the cells. It follows that the tissue culture technique applied to fresh explants, instead of to sub-cultures, can be used to determine how the metabolism of proliferating cells is influenced by various chemical and biological agents.

By applying this standardised procedure to the study of the action of insulin on chick heart explants, it has been established that the hormone has a stimulating effect on cell metabolism. Not only does the insulin increase the rate of cell proliferation and cause a greater total synthesis of protein, phospholipid, and RNA, but it also produces a distinct change in cell composition (Section III). This change involves an increase in the amount of RNAP per cell accompanied by a decrease in ASP, although the amounts of PN and LP per cell remain unaltered by the action of insulin.

Insulin can, therefore, promote increased protein, phospholipid and RNA synthesis, in addition to its known ability to stimulate the formation of fatty acids and glysogen in liver and muscle tissue. The broad character of its action suggests that insulin enables the cell to increase its energy-yielding reactions, or to couple them more efficiently to synthetic processes. Insulin is known to quicken the turnover of energy-rich phosphate bonds in ATP, and, as found in work on isolated rat diaphragm and in the present work with growing explants, insulin can significantly increase the proportion of ester phosphate at the expense of the metabolic pool of inorganic phosphate. From all the evidence available, it is clear that insulin is well qualified to act as a growth-hormone in the way originally envisaged by Mirsky (1939).

In contrast to the consistent pattern of chemical change found in the rapidly growing explants, the changes in composition in chick embryo tissues seem to be related to the extensive differentiation which accompanies development. For each organ the pattern is different (Section IV). In brain, there is a characteristic rise in RNAP and LP per cell; in muscle, ASP and PN accumulate rapidly in the cells just before hatching, when muscular activity is required to break open the shell; and, in liver, the high RNAP content of the cells is maintained, as might be expected in a tissue which, even in its adult

form, is engaged in intensive protein synthesis. In contrast, the composition of heart cells shows little change during development, and it is perhaps significant that this organ achieved its adult morphological form as early as the 8th day of incubation.

An important feature in the development of the first three organs is the pronounced and permanent increase in the protein content of their cells as they approach their functional adult form (Section IV). Only after hatching does the protein content of the heart cells show some sign of increasing in amount.

This increasing protein content of the cells presumably reflects an increase in amount and complexity of enzyme systems, and is very probably a consistent feature of cell differentiation towards the mature adult form. This view is strengthened by the observations on the chemical changes in liver during the induction of tumours; recalculation of the results of other authors in order to show amounts per cell, has revealed that the appearance of the more primitive cell types of the tumour tissue is always accompanied by a reduction in the amount of protein per cell (Section V). This involves, of course, a reduction in the activity per cell of certain, but not all of the enzyme systems. Similar reductions in protein content occur, though presumably for different reasons, in liver cells damaged by disease, malnutrition and starvation (Sections I,B & V.).

The RNA content of cells is highest when cell proliferation is most rapid or where cell function involves intense protein synthesis. In the chick heart explants, rapid and continued cell multiplication is dependent on the maintenance of a high RNAP content of the cells (Section III). When the RNAP has decreased during a period of incubation with Tyrode-serum, the normal rate of cell proliferation cannot be restored by a return to the fully adequate growth-promoting medium. Other experiments have shown that insulin can increase the RNAP content of explanted cells, an effect which is probably responsible for the more rapid cell proliferation found in the insulin-treated explants. In chick embryo heart the RNAP per cell is highest when proliferation is at its peak at 13-14 days, but in the other tissues high RNA contents are associated with intense protein synthesis, or in brain, with functional capacity (Section IV). Finally, the RNAP content is greater in the more primitive and rapidly dividing marrow cells than in the mature, differentiated leucocytes of human blood (Section V).

Of the relation between the synthesis of RNA and that of DNA, the conclusion to be drawn from this study is that DNA formation occurs only when relatively large amounts of RNA are present in the cells. This does not necessarily support an earlier concept (Brachet, 1933, 1947) that RNA acts in the sea urchin egg as a direct

precursor of DNA. In fact, all recent evidence is rather to the contrary (Schmidt, Hecht & Thannhauser, 1948; Villee, Lowens, Gordon, Leonard & Rich, 1949), and the mechanism of synthesis of DNA in the T_2 bacteriophage of E. coli is apparently quite separate from that of RNA (Cohen, 1947, 1949).

From the pattern of chemical change in the chick heart explants, it is clear that protein and phospholipid accumulation in the cells are closely linked, while the RNAP content reaches its maximal amount per cell at the same time as the ASP. The same relationships are found between the growth rates of these constituents, when they are compared by simple allometry with the growth rate of DNA. Again, there is a close relationship between ASP and RNAP in brain, heart and liver, and between PN and LP in liver tissue. In brain, development is distinguished by a steady rise in the cellular content of phospholipid, which is reflected in the higher relative growth rates found for this component. Comparison of the relative growth rates of RNAP and PN (or the ratio PN:RNAP) reveals that in all four tissues there is, during development, an increasing proportion of protein in the cells relative to the amount of RNA.

In some of the human tissues investigated, it has been possible to make direct measurements of the amounts of DNAP and RNAP per cell by chemical methods (Section V). Normal marrow cells have a higher average

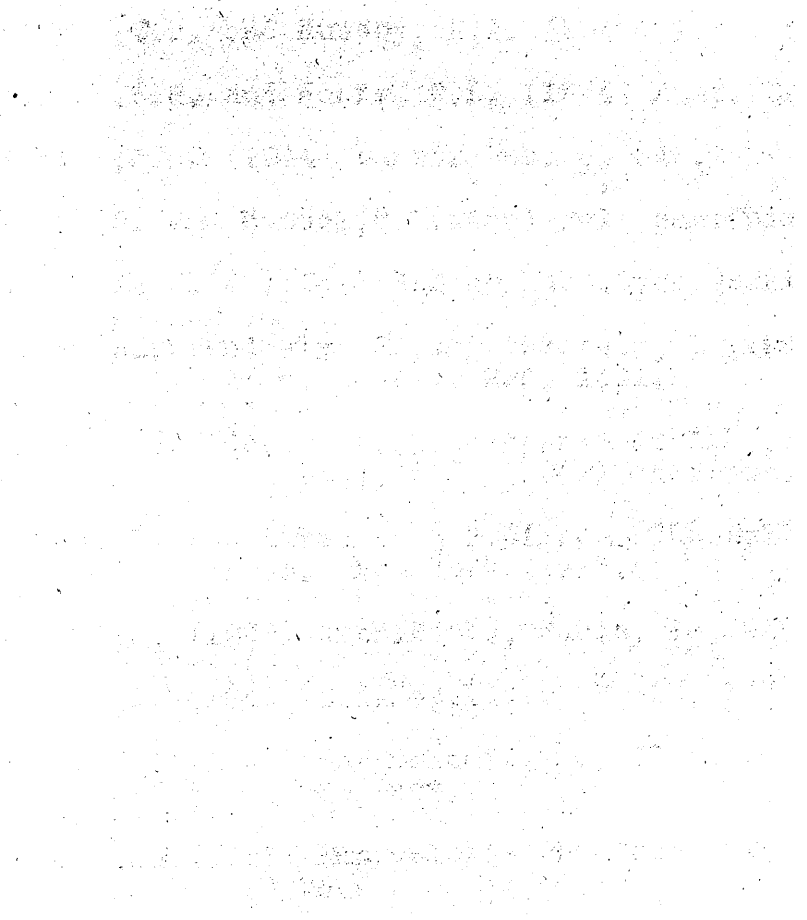
DNAP content than normal blood leucocytes, but the amounts per cell agree closely with those found in kidney and prostate. The ratio of the DNAP content of these cells to that of spermatozoa is higher than the 2:1 ratio expected between diploid and haploid cells, and it is concluded that the DNAP content of the somatic nucleus is somewhat elevated in the course of intensive cell multiplication. On the other hand, the high value for liver tissue is almost certainly caused by polyploidy, as in rodent liver.

In marrow cells from leukaemic patients, there is no change in the mean amounts of DNAP and RNAP per cell, but the means are strikingly increased in marrow cells from cases of pernicious anaemia. In particular, the extent of the increase in DNAP in the latter strongly suggests that cell maturation is blocked in this disease at a stage when the DNAP per cell is close to twice its normal diploid amount.

Although the DNAP in marrow cells is abnormally high in pernicious anaemia, the ratio RNAP:DNAP for the entire series consistently follows the RNAP content per cell in decreasing whenever cell maturation occurs, whether this happens by normal development or as the result of therapy.

In liver biopsy specimens, particularly severe losses of RNAP and PN occur in the liver cells during infective hepatitis, and the reduction per cell of these

constituents appears to run parallel with the degree of liver damage.



REFERENCES

1. Ada, G.L. (1949) Biochem., J. 45, 422.

2. Alfert, M. (1950) J.Cell.Comp. Phys. 36, 381.

3. Arnesen, K., Goldsmith, Y., and Dulaney, A.B. (1949) Cancer Res., 9, 669.

4. Avery, O.T., MacLeod, C.M., & McCarty, M. (1944) J.Exp.Med., 79, 137

5. Barber, H.N. and Callam, H.G. (1944) Nature, 153, 109.

6. Barker, G.R., Farrar, K.R. and Gulland, J.M. (1947) J.Chem.Soc., p.21.

7. Barnum, C.P. and Huseby, R.A. (1948) Arch.Biochem.,19,17.

8. Barnum, C.P. and Huseby, R.A. (1950) Arch.Biochem.,29, 7.

9. Beusley,R.R. and Hoerr, N.L. (1934) Anat. Rev.,60, 449.

10. Biesele,J.J. (1944) Cancer Res. 4, 540.

11. Bieth, R. and Mandel,P. (1950) Bull.Soc.Chim.Biol.32, 109.

12. Boivin,A. (1947) Cold Spring Harb.Symp.Quant.Biol. XII, 7

13. Boivin,A., Vendrely, R.,and Vendrely, C.(1948) Compt. rend. Acad. d.Sci., 226, 1061.

14. Borsook, H., Deasy, C.L., Haagen-Smit,A.J.,Keighley,G., and Lowy, P.H. (1949) Fed.Proc. 8, 589.

15. Boveri, T. (as cited by E.B.Wilson, The Cell, Macmillan & Co., New York, 1925.

16. Brachet,J. (1933) Arch.Biol., Paris, 44, 519.

17. Brachet,J. (1940) C.R.Soc.Biol., Paris, 133, 88.

18. Brachet,J. (1941) Enzymologia, 10, 87.(Arch.Biol.Paris, 53, 207.

19. Brachet,J. (1945) Embryologie Chemique, Desser, Liege, and Masson & Cie, Paris

20. Brachet,J. (1947) Cold Spring Harb.Symp.Quant.Biol., XII.

21. Brachet,J. (1950) Annals. N.Y. Acad.Sci. 50, 861.

22. Bradfield,J.R.G.,(1950) Biol.Rev. 25, 113.

23. Brown, G.B., Peterman, M.L. and Furst, S.S. (1948) J.biol.Chem., 174, 1043.

24. Brues, A.M. Rathbun, E.N. and Cohn, W.E. (1944) J.Cell Comp.Phys., 24, 155.

25. Bucher, O. (1940) Schweig.med.Wschr., 70, 901.

26. Caldwell, P.C., and Hinshelwood, Sir C. (1950) J.Chem. Soc., p.3156.

27. Campbell, R.M. and Kosterlitz, H.W. (1947) J.Physiol.106,12P

27a. Campbell, R.M. and Kosterlitz, H.W. (1950) J.Endocrinology, 6, 308.

28. Caspersson, T. (1936) Skand.Arch.Physiol. 73, Suppl. 8.

29. Caspersson, T. (1940) Chromosoma, 1, 562.

30. Caspersson, T. (1941) Naturwissenschaften, 29, 33.

31. Caspersson, T. (1950) "Cell Growth & Cell Function", Norton & Co. Inc., New York.

32. Caspersson, T.,Landström, H., and Aquilonais,L. (1941)

33. Caspersson, T. and Santesson (1942), Acta Radiol.Suppl.XLVI.

34. Caspersson, T. and Schultz, J. (1938) Nature, 142, 294.

35. Caspersson, T. and Schultz, J. (1939) Nature, 143, 602.

36. Caspersson, T. and Schultz, J. (1940) Proc. Nat.Acad.Sci. (U.S.) 26, 507.

37. Caspersson, T. and Thorell, B. (1941) Chromosoma, 2, 132.

38. Chantrenne, H. (1944) Enzymologia II, 213

39. Chantrenne, H. (1947) Biochim.Biophys.Acta 4, 232.

40. Chargaff, E. (1950) Experientia, 6, 201.

41. Chargaff, E. Vischer, E., Doniger, R., Green, C. and Misani, F., (1949) J.Biol.Chem., 177, 405.

42. Chargaff, E. Zamenhof, S. and Vischer, E. (1949) J.Biol. Chem. 177, 429.

43. Chernick,S.S. & Chaikoff, I.L., (1950) J.Biol.Chem.186,535.

44. Claude, A. (1938) Science, 87, 467.

45. Claude, A. (1946) J.Exp.Med. 84, 51, 61.

46. Cohen, P.P., and McGilvery, R.W. (1946) J.Biol.Chem. 166,261.

47. Cohen, S.S. (1947) Cold Spring Harb.Symp. Quant.Biol.XII,35.

48. Cohen, S.S. (1949) J.Biol.Chem., 177, 607.

49. Crossman, G. (1937) Science, 85, 250.

50. Cunningham, B. and Kirk, P.L.(1942) J.Cell Comp.Phys.20,343.

51. Cunningham, L.,Griffin, A.C., and Luck,J.M.(1950), J.Gen.Phys., 34, 59.

52. Darlington, C.D.(1948) S.E.B.Symp. 1, 252.

53. Davidson, J.N. (1945) Edin.Med.J., III, 344.

54. Davidson, J.N. (1946) J.Physiol. 105, 32P.

55. Davidson, J.N. (1947) Cold Spring Harb.Symp.Quant.Biol.XII,50.

56. Davidson, J.N. (1950) "The Biochemistry of the Nucleic Acids" 14, 1-18, Metheun(1950).

57. Davidson, J.N., Frazer, S.C., and Hutcheson, W.C. Biochem.J: in the press.

58. Davidson, J.N. and Leslie, I. (1950a) Nature, 165, 49.

59. Davidson, J.N. and Leslie, I. (1950b) Cancer Res., 10,587.

60. Davidson, J.N. and Leslie, I. (1951b) Exp.Cell Res. in the press.

61. Davidson, J.N.,Leslie, I.,Smellie,R.M.S. & Thomson,R.Y. (1950) Biochem.J.,46, Proc. XI.

62. Davidson, J.N.,Leslie,I. and Waymouth, C. (1949) Biochem. J., 44, 5.

63. Davidson, J.N.,Leslie,I. and White, J.C.(1948)J.Path. Bact. LX, 1.

64. Davidson, J.N.,Leslie,I. and White, J.C.(1950)Biochem. J.,Proc.XVI.

65. Davidson,J.N., Leslie,I. and White, J.C. (1951a) J.Path.Bact., in the press.

66. Davidson,J.N., Leslie,I. and White, J.C. (1951b) Lancet, in the press.

67. Davidson,J.N., and McIndoe, W.M. (1949) Biochem.J.47, Proc.,XVi.

68. Davidson,J.N.,McIndoe, W.M. and Smellie, R.M.S.(1951) Biochem.J.,Proc., in the press.

69. Davidson,J.N.,and Waymouth,C.(1943) Biochem.,J. 37,271.

70. Davidson,J.N. and Waymouth,C.(1944a) Biochem.J.,38,39

71. Davidson,J.N. and Waymouth,C.(1944b) Biochem.J. 38,375.

72. Davidson,J.N. and Waymouth,C.(1944-1945)Nutr.Abstr.& Rev. 14,1.

73. Davidson,J.N. and Waymouth,C.(1944c)Biochem.J.38,379.

74. Davidson,J.N. and Waymouth,C.(1945) Biochem.J.39,188.

75. Davidson,J.N. and Waymouth,C.(1946) Biochem.J.40,568.

76. Davison,K.B. and Osgood,E.(1951)Results quoted by Mirsky and Ris (1951)

77. Di Stefano,H.S.,(1948) Proc.Nat.Acad.Sci.(U.S.)34,75.

78. Dounce, A.L. (1943) J.Biol.Chem. 147, 685

79. Dounce,A.L. (1950) Annals N.Y. Acad.Sci., 50, 982.

80. Elwyn, D. & Sprinson,D.B.(1950) J.Am.Chem.Soc. 72,3317.

81. Entenman, C.Chaikoff,I.L. and Zilversmit (1946) J.Biol.Chem. 166, 15.

82. Ephrussi, B. and Hugues,Y.(1930) C.R.Soc.biol.,105,697

83. Feulgen,R. and Rossenbeck,H.(1924) Hoppe-Seyl.,135,203.

84. Fischer,A. (1946) "Biology of Tissue Cells",(Copenhagen)

85. Fisher, R.A. (1950) "Statistical Methods for Research Workers", Oliver & Boyd, London.

86. Forker,L.L.,Chaikoff,I.L.Entenman,C. and Tarver,H.(1951) J.Biol.Chem. 188, 37

87. Friedberg, F., Schulman, M.P. and Greenberg, D.M. (1948) J.Biol.Chem. 173, 437.

88. Furst, S.S., Roll.P.M. & Brown, G.B. (1950) J.Biol.Chem. 183, 251.

89. Gersh, I. (1943) see Hirsky, A.E. (1943) Advances in Enzymol. 3, 1.

90. Gjessing, E.C., Floyd, C.S. and Chanutin, A. (1951) J.Biol.Chem., 188, 155.

91. Gopal-Ayengar, A.R. & Cowdry, E.V., (1947) Cancer Res. 7, 1.

92. Goranson, E.S. and Erulkar, S.D. (1949) Arch.Biochem. 24, 40.

93. Griffin, A.C., Cook, H. and Cunningham, L. (1949) Arch. Biochem., 24, 190.

94. Griffin, A.C., Nye, W.N. Noda, L. and Luck, J.M. (1949) J.Biol.Chem. 176, 1225.

95. Gulland, J.M., Barker, G.R. and Jordan, D.O. (1945) Ann.Rev.Biochem., 14, 175.

96. Hahman, J.W. (1950) Exp.Cell Res. 1, 382.

97. Hangaard, N., Marsh, J.B. and Stadie, W.C. (1951) J.Biol. Chem. 189, 59.

98. Hertwig, G. (1934) Z. ind. Abst. Vererbg. 70, 496.

98a. Hertwig, G. (1939) Z. Milar, Anat. Forschg. 45, 37.

99. Hevesy, G.C.V., (1945) Nature, 156, 534

100. Hochberg, I. and Hyden, H. (1949) Acta Physiol.Scand. 17, Suppl.60.

101. Hogeboom, G.H. (1949) J.Biol.Chem., 177, 847.

102. Hogeboom, G.H. and Schneider, W.C. (1950) J.Nat.Canc. Inst. 10, 983.

103. Howard, A. and Pelc, S.R. (1950) Brit.J.Radiol. 23, 634.

104. Hull, W. and Kirk, P.L. (1950a) J.Gen.Phys. 33, 327.
" " " " (1950b) J.Gen.Phys. 33, 335.
" " " " (1950c) J.Gen.Phys. 33, 343.

105. Hultin, T. (1950) Exp.Cell Res., 1, 376.

106. Hyden, H. (1943) Acta.Physiol.Scand. 6, Suppl.XVII.

107. Hyden, H. (1947) Cold Spring.Harb.Symp.Quant.Biol.X11,115.

108. Illing, G. (1905) Anat.Anz. 26, 177.

109. Jacobj, W. (1925) Arch. Entwicklungsmech. Organ,106,124.

110. Jeener, R. (1947) Actualites Biochimiques, 10, 88

111. Jeener, R. and Szarfarz, D. (1950) Experentia, V1, 60.

112. Johnson, R.B. and Bloch, K. (1951) J.Biol.Chem.,188,221

113. Jones, W. (1920) "The Nucleic Acids", Longmans Green & Co. London.

114. Jorpes, E. (1924) Biochem. Ztschr. 151, 227.

115. Jorpes, E. (1928) Acta Med.Scand. 68, 253, 503.

116. Kennedy, E.P. and Lehninger, A.L. (1949) J.Biol.Chem. 179,957.

117. Klein, E.,and Klein, G. (1950) Nature, 166, 833.

118. Klein, E., Kurnick, N.B. and Klein, G. (1950) Exp. Cell Res. 1, 127.

119. Koller, P.C. (1947) Symp.Soc.Exp.Biol. 1, 270.

120. Kosterlitz, H.W. (1947) J.Physiol. 106, 194.

121. Lagerstedt, S. (1949) Acta Anat. Suppl.IX.

122. Lan, T.H. (1943) J.Biol.Chem. 151, 171.

123. Latta, J.S. and Bucholz, D.J. (1929) Arch.exper. Zellforsch. XXlll, 146.

124. Lepage, G.A. and Heidelberger, C. (1951) J.Biol.Chem. 188, 593.

125. Leslie, I. and Davidson, J.N. (1951) Biochim.Biophys. Acta., in the press.

126. Leuchtenberger, C., Vendrely, R. and Vendrely, C. (1951) Proc.Nat.Acad.Sci.(U.S.) 37, 33.

127. Levene, P.A. and Mori, T. (1929) J.Biol.Chem.83, 803.

128. Levine, H.B. (1950) J.Gen.Phys. 34, 161.

129. Levy, M. and Palmer, A.H. (1943) J.Biol.Chem.150, 271.

130. LeBreton, E. and Schaeffer, G. (1923) Trav.Inst.Phys. Univ.,Strasbourg.

131. Linderstrøm-Lang, K. (1939) Harvey Lect. 34, 214.

132. Linderstrøm-Lang, K. (1949) Exp.Cell Res., Suppl.I.

133. Lipmann, F. (1945) J.Biol.Chem. 160, 173.

134. Lipmann, F. and Fischer, A. (1932) Biochem.Zscht. 244, 187.

135. Lotspeich, W.D. (1949) J.Biol.Chem. 179, 175.

136. McCarty, M. & Avery, O.T. (1946) J.Exp.Med. 83,97.

137. Ma, T.S., and Zuazaga, G. (1942) Ind.Eng.Chem. 14, 280.

138. Mark, D.D., and Ris, H. (1949) Proc.Soc.Exper.Biol. & Med. 71, 727.

139. Marshak, A. (1941) J.Gen.Physiol. 25, 275.

140. Mandel, L. Jacob, M. and Mandel, P. (1950) C.R.Soc. Biol. CXLIV, 275.

141. Mandel, P. Jacob, M. & Mandel, L. (1949) C.R. l'Acad. Sci., 299, 1370.

142. Mandel, P. Jacob, M. & Mandel, L. (1950) Bull.Soc. Chim.Biol. 32, 80.

143. Mandel, P. and Bieth, R. (1950) XVIIIth Intern.Phys. Congr.Abs. 350.

144. Mandel, P., Metais, P. and Cuny, S. (1950) C.R. Acad. Sci.,Paris, 231, 1172.

145. Metais, P. and Mandel, P., (1950) C.R. Soc.Biol. CXLIV, 277.

146. Minor, A.H., and Burnett, L. (1948) Blood, 3, 799.

147. Minot, G.S. (1908) "The Problem of Age, Growth and Death", London.

148. Mirsky, I.A. (1938) Am.J.Physiol. 124, 569.

149. Mirsky, I.A. (1939) Endrocrinology, 25, 152.

150. Mirsky, A.E. and Pollister, A.W. (1946) J.Gen.Physiol, 30,117.

151. Mirsky, A.E. and Pollister, A.W. (1947) Cold Spring Harb. Symp. Quant.Biol.XII.

152. Mirsky, A.E. and Ris, H. (1947) J.Gen.Physiol. 31, 1 & 7.

153. Mirsky, A.E. and Ris, H. (1949) Nature, 163, 666.

154. Mirsky, A.E. and Ris, H. (1951) J.Gen.Physiol.34, 451.

155. Mitchell, J.S. (1942) Brit.J.Exptl. Path. 23, 285,296,309.

156. Moog, F. and Steinbach, H.B. (1946) J.Cell.Comp. Physiol. 28, 209.

157. Moog, F. (1947) J.Exptl. Zool. 105, 209.

158. Muntwyler, E., Seifter, S., and Harkness, D.M. (1950) J.Biol.Chem.,184, 181.

159. Needham,J. (1931) Chemical Embryology, Cambridge University Press.

160. Needham,J. (1932) Nature, 130, 845.

161. Needham,J. (1934) Biol.Rev. 9, 79.

162. Needham,J. (1942) Biochemistry and Morphogenesis, C.U.P.

163. Needham,J. and Needham,D.M. (1930) J.Exp.Biol.7, 317.

164. Ogur, M. and Erickson, P.O. (1950) Fed.Proc. 9, 210.

165. Ogur, M. and Erickson, R.O., Rosen, G.U.,Sax, K.B., Holden, C. (1951) Exptl.Cell Res. II, 73.

166. Ogur, M. and Rosen, G. (1950) Arch.Biochem. 25, 262.

167. Ostern, P., Terszakowec, J. and Hubl. S., (1938) Hoppe-Seyl. Z. 272, 87.

168. Pasteels, J. & Lison, L. (1950a) C.R.Acad.Sci., 230,780.

169. Pasteels, J. & Lison, L. (1950b) Arch.Biol.LXI, 445.

170. Pasteels, J. & Lison, L. (1950c) Bull.l'Acad.Roy.Belg., XXXVI, 348

171. Pasteels, J. & Lison, L. (1950) Arch.Biol. LXII, 1.

172. Plimmer, R.H.A. and Scott, F.H. (1908) J.Chem.Soc. 93,1699.

173. Pollister, A.W. and Leuchtenberger, C. (1949) Nature, 163, 360.

174. Pollister, A.W. and Ris, H. (1947) Cold Spring Harb. Symp. Quant.Biol. XII.

175. Popjak, G. and Beekmans, H.L. (1950) Biochem.J.47, 233.

176. Price, J.M. and Laird, A.K. (1950) Cancer Res. 10, 650.

177. Price, J.M.,Miller, E.C. and Miller, J.A.(1948) J.Biol. Chem. 173, 345.

178. Price, J.M.,Miller, E.C.,Miller, J.A. and Weber,G.M. (1950) Cancer Res. 10,18.

179. Reichard, P. (1949) Acta Chem.Scand. 3, 422.

180. Reichard, P. & Estborn, B. (1951) J.Biol.Chem.188,839.

181. Richards, O.W.,& Kavanagh, A.J. (1945) "Growth and Form" Clarendon Press, Oxford.

182. Ris, H. & Mirsky, A.E. (1949) J.Gen.Physiol. 33,125.

183. Sacks, J. (1945) Am.J.Physiol., 143, 157.

184. Sanford, K.,Earle,W.R. and Likely, D.G. (1948) J.Nat. Canc.Inst. 9, 229.

185. Schmidt, G. and Thannhauser,S.J. (1945) J.Biol.Chem. 161, 83.

186. Schmidt, G. Hecht, L., and Thannhauser, S.J. (1948) J.Gen.Physiol.

187. Schneider, W.C. (1945) J.Biol.Chem. 161, 293.

188. Schneider, W.C. (1946) Cancer Res. 6, 685.

189. Schneider, W.C. and Hogeboom,G.H. (1950a) J.Nat.Canc. Inst. 10, 969.

190. Schneider, W.C. and Hogeboom,G.H. (1950b),J.Biol.Chem. 183, 123.

191. Schneider, W.C., Hogeboom,G.H., and Ross, H.E.(1950) J.Nat.Canc. Inst. 10, 977.

192. Schneider, W.C. and Potter,V.R. (1949) J.Biol.Chem. 177, 898.

193. Schrader, F., and Leuchtenberger, C.(1950) Exptl. Cell.Research,I,421.

194. Schoenheimer, R. (1942) "The Dynamic State of Body Constituents",London.

195. Seifter, S.,Muntwyler,E. and Harkness,D.M.(1950), Proc.Soc.Exp.Biol.Med. 75,46.

196. Speck, J.F.,(1949) J.Biol.Chem. 179, 1405.

197. Spiegelman, S. and Kamen, M.D.,(1946) Science, 104,581.

198. Stedman, E. and Stedman, E.(1943) Nature,152,267.

199. Stedman, E. and Stedman, E.(1947) Cold Spring Harb. Symp.Quant.Biol.,XII.

200. Stedman, E. and Stedman, E.(1950) Biochem.J. 47, 508.

201. Steinbach, H.B. and Moog, F. (1945) J.Cell Comp. Physiol. 26, 175.

202. Stern, K.G. (1947) Biol.Symp.10, 291.

203. Stoneburg, C.A. (1939) J.Biol.Chem. 129, 189.

204. Stowell, R.E. (1947) Symp.Soc.Exp.Biol. 1, 190.

205. Swift, H.H. (1950a) Phys.Zool. XXIII , 169.

206. Swift, H.H. (1950b) Proc.Nat.Acad.Sci. 36, 643.

207. Taylor, H. (1949) J.Exptl.Med. 89, 399.

208. Teissier, G. (1931) Trav.Stat.Biol.Roscoff, 9, 27.

209. Thorell, B. (1944) Acta.Med.Scand. 117, 334.

210. Thorell, B. (1947) Acta.Med.Scand.Suppl. CC.

211. Tompkins, E.R., Cunningham, B. and Kirk, P.L. (1947) J.Cell.Comp.Physiol. 30, 1.

212. Tulasne, R., and Vendrely, R. (1947) C.R. Soc.Biol. Paris, 141, 674.

213. Vendrely, R. and Vendrely, C. (1948) Experentia, 4, 434.

214. Vendrely, C. and Vendrely, R. (1949a) C.R.Soc. Biol.CXLIII, 1386.

215. Vendrely, R. and Vendrely, C. (1949b) Experentia, 8, 327.

216. Vendrely, R. and Vendrely, C. (1950) Compt.rend. Paris, 230, 670.

217. Villee, C.A., Deane, H.W. and Hastings A.B.,(1949) J.Cell.Comp.Physiol. 34, 159.

218. Vischer, E. and Chargaff, E. (1948) J.Biol.Chem. 176, 715.

219. Vischer, E., Zamenhof, S., and Chargaff, E. (1949) J.Biol.Chem. 177, 429

220. Waymouth, C. (1949) Exptl.Cell.Res.,Suppl.1.,

221. Willmer, E.N. (1933) J.Exp.Biol.10, 317, 323, 340.

222. Willmer, E.N. (1935) "Tissue Culture", London.

223. Willmer, E.N. (1942) J.Exptl.Biol. 18, 237.

224. Willmer, E.N. (1945) "Growth and Form" Clarendon Press, Oxford.

225. Wilson, A., Jackson, E.B., and Brues, A.M. (1942) J.Gen.Physiol. 25, 689.

226. Wyatt, G.R. (1951) Biochem. J., 48, 584.