Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

3. METHODS FOR THE DISRUPTION OF KREBS II MOUSE-ASCITES-TUMOUR CELLS

By E. M. MARTIN, J. MALEC,* J. L. COOTE AND T. S. WORK
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 8 February 1961)

We have reported on the changes in nucleic acid and protein metabolism in Krebs II ascites-tumour cells during the growth of encephalomyocarditis virus in this cell (Martin, Malec, Sved & Work, 1961). For further work, it was decided to study the metabolic changes in different subcellular fractions of the Krebs cell. Before this could be done, however, methods had to be devised for the rupture of Krebs cells and for the quantitative separation of the subcellular particles.

The Krebs II mouse carcinoma in the ascitic form (Klein & Klein, 1951) is mechanically an exceptionally tough cell and is quite undamaged by homogenizing in a Potter-Elvehjem homogenizer with either sucrose or inorganic buffers. Our objective was to disrupt with as little damage as possible to subcellular components. Damage was assessed by three independent criteria: firstly, the microscopic appearance of the products, secondly, the distribution of deoxyribonucleic acid, ribonucleic acid and protein in separated nuclei, mitochondria, microsomes and cell sap, and thirdly, the capacity of the microsomal fraction to incorporate amino acids into protein. The third criterion is admittedly selective and arbitrary but it was of special interest in our investigations on virus biosynthesis.

METHODS

Homogenizers. Two types of homogenizer were used. The Potter-Elvehjem homogenizer consisted of a 15 ml. capacity, round-bottomed centrifuge tube fitted with a polytetrafluoroethylene (Teflon) pestle, with a clearance of about 0.2 mm. between pestle and the glass tube. The second homogenizer was made of glass according to the instructions of Dounce (1955) and will be referred to throughout as the Dounce homogenizer. In this homogenizer the clearance between the glass ball and the tube wall was only about 0.03 mm. on a 1.2 cm. diameter precision bore glass tube.

Ultrasonics oscillator. A Mullard laminated nickel magnetostriction transducer was brazed to a stainless-steel cup of 2 cm. diameter and 7.5 cm. length. This was coupled to an oscillator with an output of 200 w and tuned to 25 kcy./sec. The apparatus is similar to that described by

Davies (1959). When in use, the steel cup was surrounded by crushed ice and partially filled with ice-water. The cell suspension (2 ml.) in a thin-walled plastic tube (Spinco tube) was immersed in the ice-water.

Analyses of cell fractions. The methods were those described by Martin *et al.* (1961). Where necessary, the preferred method is indicated in the appropriate Table.

Assay for radioactivity. Proteins were isolated, estimated and assayed for radioactivity as described by Martin *et al.* (1961). Randomly labelled [^{14}C]amino acids (The Radiochemical Centre, Amersham, Bucks) were used throughout.

Special chemicals. Phosphoenolpyruvate, 3-phosphoglycerate and pyruvate kinase were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany.

Assay of succinoxidase. The succinoxidase activity of various cell fractions was measured in a Warburg apparatus according to the method of Schneider & Potter (1943).

RESULTS

Ultrasonic oscillator

Krebs II mouse-ascites cells were washed in phosphate-buffered saline as described by Martin *et al.* (1961), packed by centrifuging and resuspended in the media listed in Table 1. The suspensions were exposed to the sonic oscillator for various times and samples were withdrawn for microscopic examination in nigrosin-potassium chloride (Martin *et al.* 1961). With this dye, nuclei appear as uniformly stained disks free of cytoplasm, while undamaged cells are unstained. It was clear from the results, that the time of exposure to ultrasonics was highly critical and that magnesium chloride had a considerable stabilizing influence; indeed, preparations in pure sucrose were gelatinous, indicating that the DNA had been released from the nuclei.

Although it was evident that ultrasonics were unlikely to give a preparation containing 100% undamaged nuclei, the sucrose-magnesium chloride-tris mixture looked promising and was further investigated. Washed ascites cells were suspended in this medium, disintegrated, centrifuged and the precipitate was discarded. The supernatant was incubated with [^{14}C]valine under the conditions

* Inst. Hematologii, Chocimska 5, Warszawa, Poland.

given in Table 2 and the radioactivity of the protein was then measured. The results indicated that, although ultrasonics tended to break nuclei, the microsome-cell sap fraction was active in protein biosynthesis. However, the degree of nuclear disruption was difficult to control and alternative methods of cell disruption were therefore investigated.

Dounce homogenizer

When Krebs II mouse-ascites-tumour cells were suspended in sucrose-calcium chloride (0.25 M-5 mM) and homogenized as described by Dounce (1955) the preparation had excellent morphology with almost 100% breakage of the cells and few damaged nuclei but the microsome-cell sap fraction did not incorporate [14 C]valine. When magnesium chloride (0.01 M) was substituted for calcium chloride excellent incorporation of [14 C]valine into the microsome-cell sap fraction was obtained and the free nuclei were clean and appeared to be undamaged. About 70% cell breakage was obtained before any significant number of damaged nuclei appeared. The activity in protein synthesis was closely similar to that of ultrasonic microsomes prepared from another sample of the same batch of cells (Table 3). In this experiment, the addition of soluble RNA did not greatly increase the rate of incorporation but did prolong the period of incorporation.

Analytical composition of subfractions of Dounce homogenate

Freshly collected ascites-tumour cells were homogenized in sucrose-magnesium chloride medium and separated by differential centrifuging into four fractions consisting of nuclei, mitochondria, microsomes and cell sap. Each fraction was analysed for total nucleic acid, DNA and protein (Table 4). In this experiment, an attempt was made to carry the homogenizing treatment to the point where no whole cells remained. Although it was easy to obtain 70% disruption, 100% disruption could not be achieved without damage to nuclei as indicated by appearance of DNA in fractions other than the nuclear fraction.

Disruption of cells by homogenizer after osmotic shock

Since neither ultrasonics nor the high shear of a Dounce homogenizer produced complete cell disruption without damage to nuclei, it was decided to try the combined effect of osmotic shock and either the Dounce- or the Potter-type homogenizer. Conditions were worked out for the successful application of either homogenizer but the conditions were quite different for the two types of instrument. As each had advantages, they will be described separately. Severe osmotic shock coupled with gentle homogenizing in the Potter homogenizer gave a preparation that was morphologically and analytically most satisfactory, but mild osmotic shock followed by severe homogenization in the Dounce homogenizer gave a preparation that was more effective in tests of capacity for protein biosynthesis.

Table 1. *Disintegration of Krebs II mouse-ascites-tumour cells by ultrasonics*

The percentage of morphologically intact free nuclei was estimated by inspection and counting in a Neubauer cell after dilution with nigrosin (0.2%) in KCl (0.3 M). The method of disintegration is described in the text. Tris-HCl buffer, pH 7.5, is referred to as tris.

Suspending medium (1×10^8 cells/ml.)	Duration of exposure to ultrasonics (sec.)	Percentage undamaged free nuclei
0.25 M-Sucrose	20	None
	30	None
	40	20
	50	60
	60	Nearly all nuclei broken
0.25 M-Sucrose, 0.05 M-tris	40	20
	50	60
	60	Nearly all nuclei broken
0.25 M-Sucrose, 5 mM-MgCl ₂	50	None
	60	5
	75	15
0.25 M-Sucrose, 0.05 M-tris, 5 mM-MgCl ₂	40	10
	50	50
	60	70 (some broken nuclei)

Table 2. *Incorporation of [14 C]valine into the microsome-cell sap fraction of Krebs II ascites-tumour cells after disintegration by ultrasonics*

Washed cells (3×10^8) were suspended in 2 ml. of medium with components at the following concentrations: sucrose, 0.25 M; KCl, 0.025 M; MgCl₂, 5 mM; tris buffer, pH 7.5, 0.05 M. After disintegration by ultrasonics (50 sec.) the homogenate was centrifuged (10 000 g for 10 min.) and the supernatant incubated for 30 min. at 37° with 2 μ C of [14 C]valine and the energy source indicated. PGA, Potassium salt of 3-phosphoglyceric acid; ATP, potassium salt of adenosine triphosphate.

Energy source	Radioactivity of protein* (μ mC/g.)
ATP, 10 μ moles	14
ATP, 10 μ moles + PGA, 15 μ moles	76
ATP, 5 μ moles + PGA, 33 μ moles	115
PGA, 33 μ moles	294

* The microsomes were not isolated after incubation and this material was a mixture of cell-sap protein and microsomal protein (cf., however, Table 3).

Potter homogenizer

It was noted (Table 1) that Mg^{2+} ions have a considerable stabilizing effect on Krebs cells and in any buffer that contained Mg^{2+} ions the cells were completely resistant to the Potter homogenizer. If, however, cells were washed in buffer free of Ca^{2+} or Mg^{2+} ions (solution A of Martin *et al.* 1961, diluted with one-quarter of its own volume of water), they became less stable and could then be disrupted by a combination of osmotic shock and gentle homogenizing. The most successful method is given below.

Table 3. *Incorporation of [^{14}C]valine into microsomal protein during incubation with cell sap and soluble ribonucleic acid*

Microsome-cell sap mixtures were prepared by ultrasonic disintegration as in Table 2 or by the Dounce homogenizer as described in the text. Each flask contained microsomes and cell sap from 2×10^8 cells, $2 \mu C$ of [^{14}C]valine and 20μ moles of potassium phosphoglycerate in a final volume of 3.7 ml. Other components were present in the following final concentrations: sucrose, 0.25M; tris buffer, pH 7.5, 0.05M; $MgCl_2$, 5 mM. After the stated period of incubation excess of [^{14}C]valine in sucrose (0.25M) was added and the microsomes were spun down at 100 000g. Protein was isolated in the usual way.

Components	Incubation time (min.)	Radioactivity of microsomal protein (μ mc/g.)
Dounce microsomes + cell sap	0	24
	20	924
	40	840
Sonic microsomes + cell sap	0	23
	20	888
	40	880
Dounce microsomes + cell sap + soluble RNA (1.0 mg.)	20	960
	40	1230*

* Soluble RNA was prepared from Krebs cells by the method described by Allen, Glassman & Schweet (1960).

Freshly collected Krebs ascites-tumour cells were washed twice with phosphate-buffered saline (Martin *et al.* 1961) and once with diluted solution A. The cells were resuspended in sucrose-potassium chloride (0.125M–0.075M) and counted. About 3×10^8 cells were spun at 2200g for 5 min. at 0° and the cell volume (V_1) was noted. The supernatant was replaced by ice-cold water ($5V_1$) and the cells were resuspended. The suspension was immediately recentrifuged at 0° (2200g for 3 min.) and the cell volume (V_2) noted. The supernatant was discarded and replaced by ice-cold water ($11V_1 - V_2$). The cells were resuspended by gentle hand homogenizing and, after 5 min. at 0°, sucrose (1.5M) was added to a final concentration of 0.1M. After a further 10 min. at 0° the suspension was vigorously homogenized with the Potter homogenizer (3000 rev./min. for 15 sec.) and sucrose-potassium chloride (0.75M–0.5M) was immediately added to a final potassium chloride concentration of 0.1M. This method gave about 85% cell rupture and clean undamaged nuclei.

Isolation of subcellular fractions

The whole cells and nuclei were spun down together, resuspended in sucrose (0.3M) containing EDTA (sodium salt; 0.04M; adjusted to pH 7.3 with N-potassium hydroxide), washed once with this mixture, centrifuged at 2200g for 5 min., and resuspended in ice-cold water ($8V_1$) by gentle hand homogenizing. Sucrose (1.5M) was added at once to a final concentration of 0.1M and after 10 min. at 0° the preparation was homogenized in the Potter homogenizer (3000 rev./min. for 15 sec.). Sucrose-potassium chloride (0.75M–0.5M) was immediately added as before and the nuclei were collected by spinning at 450g (5 min.). The nuclei were now hardly contaminated by whole cells. The overall yield of nuclei was about 85% of the number of cells taken.

Table 4. *Distribution of deoxyribonucleic acid, total nucleic acid and protein in subfractions of an ascites-tumour-cell homogenate prepared in a Dounce homogenizer with sucrose-magnesium chloride*

Washed tumour cells (2×10^8) were suspended in 3 ml. of sucrose- $MgCl_2$ (0.25M–0.01M), cooled to 0°, and homogenized with about 200 strokes of a Dounce homogenizer as described in the text. The homogenate was spun at 700g for 5 min. The pellet was resuspended in 20 ml. of sucrose (0.25M) and recentrifuged at 700g. The combined supernatants were spun at 700g for 5 min. and the pellet (intermediate) was analysed separately. The sucrose supernatants were recentrifuged first at 6000g for 20 min. (mitochondria) and then at 100 000g for 60 min. (microsomes). All fractions were resuspended in 0.9% NaCl and, together with the final supernatant, were treated with $HClO_4$ (final concentration 0.5M). Total nucleic acid, DNA and protein were estimated as described by Martin, Malec, Sved & Work (1961).

Fractions	Total nucleic acid (μ g. of P/ 10^8 cells)	DNA (μ g. of P/ 10^8 cells)	Protein (mg./ 10^8 cells)
Nuclei + whole cells	214	193	4.9
Intermediate	5.5	3.8	0.21
Mitochondria	35.4	7.2	1.96
Microsomes	114	0.48	2.16
Cell sap	31.6	0	5.40

Table 5. *Distribution of deoxyribonucleic acid, ribonucleic acid, protein and succinoxidase in subcellular fractions obtained from Krebs II ascites-tumour cells by osmotic shock*

The methods of disruption and analyses are described in the text. The fraction referred to as intermediate is material which spun down at 700g after the nuclei had been removed from the main homogenate. Percentage recovery in each case is based on analysis of the whole lysate after the initial disruption.

Fraction	Protein		RNA		DNA		Succinoxidase	
	mg./10 ⁸ cells	%	μg. of P/ 10 ⁸ cells	%	μg. of P/ 10 ⁸ cells	%	μl. of O ₂ / 10 ⁸ cells/hr.	%
Nuclei	1.43	8.7	26.7	11.7	193	100	6	0.7
Intermediate	0.16	0.97	2.9	1.2	0.8	0.5	23	2.5
Mitochondria	2.27	13.8	35.8	15.7	0	0	937	102
Microsomes	2.72	16.5	139	61.1	0	0	0	0
Cell sap	5.52	33.5	26.4	11.6	0	0	0	0
Percentage recovery	—	73.5	—	102.3	—	100.5	—	105.2

Mitochondria were separated from the combined cytoplasmic fractions by centrifuging (10 000g), washed by resuspension in sucrose-potassium chloride (0.25M–0.1M) and recentrifuged.

Microsomes were collected at 100 000g for 1 hr. in a Spinco preparative centrifuge. They were not washed. Cell sap was taken to be the material that did not spin down at this speed.

Distribution of succinoxidase, deoxyribonucleic acid, ribonucleic acid and protein in subcellular fractions prepared by osmotic shock

As an index of the effectiveness of the above method of cell rupture the different fractions were analysed for DNA, RNA and protein by the methods used earlier (Martin *et al.* 1961), and in addition all fractions were assayed for succinoxidase activity; the results are given in Table 5. Since virtually all the DNA was found in the nuclear fraction and all the succinoxidase in the mitochondrial fraction the method is apparently satisfactory. This conclusion is reinforced by the presence of over 60% of the RNA in the microsomal fraction. The amount of RNA that has been leached out of the nuclei cannot be large since the ratios of RNA to DNA and of protein to DNA are within the range usually found for nuclei prepared by non-aqueous techniques (Allfrey, Mirsky & Stern, 1955). Also, the value for RNA in the cell sap is low. It is reasonable to suppose that if any significant quantity of RNA had been leached out of the nuclei it would have appeared in this fraction. The rather low recovery of protein in these experiments (Table 5) is probably explained by the physical characteristics of the nuclear proteins. The method of isolation of protein (Martin *et al.* 1961) involved extraction of the nuclei with hot perchloric acid. Further losses occurred when the acid-extracted nuclei were extracted with alkali since the nuclei contained material (probably glycoprotein) insoluble in alkali (cf. Dallam, 1954). The almost complete absence of succinoxidase from the nuclear fraction indicates that it is free

from cytoplasmic or whole-cell contamination, and the complete absence of this enzyme complex from the microsomal fraction indicates that the mitochondria have not been disrupted.

Protein biosynthesis by osmotic microsomes

The obvious analytical advantages of osmotic shock combined with the Potter homogenizer are accompanied by certain disadvantages with respect to protein biosynthesis in cell-free preparations. First, the cell sap is too dilute to be used as a suspending medium for the microsomes, and, secondly, even when concentrated cell sap is prepared by other methods and combined with these microsomes, the rate of incorporation is lower than that achieved with the Dounce homogenate. In an attempt to overcome the disadvantages of both methods further experiments were made in which mild osmotic shock was combined with the use of a Dounce homogenizer. The conditions which were most successful in producing a high disruption rate, a limited degree of nuclear damage and a microsome fraction with a high capacity for amino acid incorporation are set out in Table 6. This method is preferable to the original Dounce method or the magnesium chloride-sucrose Dounce method in that it is very much more rapid but it does not give such effective separation between subcellular fractions as can be obtained by the more rigorous double-osmotic-shock method. Analyses of the various subcellular fractions obtained by this combination of osmotic shock and the Dounce homogenizer are given in Table 7. Although nearly all the DNA was found in the nuclear fraction, the proportion of protein found in this fraction was too high presumably because the method did not produce nuclei free from adherent cytoplasm. Nevertheless, this method was adopted as the most suitable for rapid preparation of a microsome-cell sap system capable of protein biosynthesis.

It will be noticed in comparing Tables 4, 5 and 7 that the total DNA per 10⁸ cells varies over the range 193–228 μg. of DNA phosphorus. This

variation is within the range of the sampling and counting error in the Neubauer cell and does not necessarily indicate variability in the DNA content of successive Krebs cell preparations. It might be permissible to adjust the figures in all four tables to a common DNA value but we have insufficient evidence to justify this.

Osmotic shock potentiated by glycerol

Since the methods of osmotic shock described above resulted in a very dilute solution of cell sap, a further attempt was made to overcome this disadvantage by potentiating the osmotic shock with the aid of glycerol. It is known from the work of Lovelock (1954) and others that glycerol passes freely across the membranes of mammalian cells. Krebs II ascites-tumour cells were therefore washed in Ca^{2+} - and Mg^{2+} -free phosphate buffer and incubated with 15% glycerol at various temperatures and for various times. In the early stages of incubation the cell wall was heavily reticulated and the cell size greatly reduced but after 30 min. at 26° or 5 min. at 37° the cells had returned to normal size. The cells were spun down and

submitted to osmotic shock by resuspension in water.

The treatment gave effective disruption but the separation between cell components was not as clean as with the more elaborate double-osmotic-shock method. The percentage of protein in the nuclear fraction was too high and that in the mitochondrial and microsomal fractions too low. These results suggest that cytoplasmic debris had clung to the nuclei. Attempts to improve the method were not pursued since microsomes prepared by glycerol-potentiated osmotic shock had only about one-eighth the amino acid-incorporating activity of Dounce-magnesium chloride microsomes in the usual test with [^{14}C]valine.

DISCUSSION

The results presented in this paper emphasize the difficulty encountered in the preparation of undamaged nuclei, mitochondria and microsomes from the Krebs II ascites-tumour cell. This cell appears to be exceptionally robust, and methods which are successful with the Ehrlich ascites-tumour cell (Littlefield & Keller, 1957) disrupt only a small percentage of Krebs cells. The use of the Dounce homogenizer, combined with a form of osmotic shock, has been described by Hawtrey & Silk (1960) and by Sauer, Martin & Stotz (1960) for the rupture of Ehrlich cells. It is difficult to judge the efficacy of either of these methods, however, since neither group of authors has published a full analysis of their subcellular fractions.

McComb & Yushok (1959) have successfully disrupted Krebs cells in a mixture of sucrose and EDTA. This method obviously depends on the complete removal of Ca^{2+} and Mg^{2+} ions, but cells disrupted in this way have given, in our hands, microsome-cell sap preparations that are incapable of protein biosynthesis.

The preparation of clean nuclei from the crude 700g precipitate obtained from the first lysis in the double-osmotic-shock method involves the selective rupture of undamaged cells, previously treated with an agent forming a complex with a bivalent cation, when suspended in dilute sucrose solutions.

Table 6. *Incorporation of [^{14}C]valine into microsome protein by Krebs II ascites-tumour cells disrupted in a Dounce homogenizer after mild osmotic shock*

Cells were washed in phosphate-buffered saline, centrifuged at 0° and 2300g for 3 min. and the packed cell volume (*V*) was noted. Cells were resuspended in 0.01 M- MgCl_2 (3*V*) at 0° and after 2 min. they were disrupted by 10–20 strokes of the Dounce homogenizer. Sucrose (1.5 M) was added immediately to a final concentration of 0.25 M and the homogenate spun at 6000g for 15 min. The supernatant (2 ml.) was incubated with [^{14}C]valine (2 μC) together with potassium phosphoglycerate (20 μmoles), salts and buffer as indicated in Table 3. A second sample of the same batch of cells was disrupted in sucrose- MgCl_2 as described in Table 3.

Medium for disruption	Incubation time (min.)	Radioactivity of protein ($\mu\text{mC/g.}$)
MgCl_2	0	29
Sucrose- MgCl_2	0	30
MgCl_2	40	1150
Sucrose- MgCl_2	40	1200

Table 7. *Distribution of deoxyribonucleic acid, total nucleic acid and protein in subfractions of an ascites-cell homogenate prepared with a Dounce homogenizer after mild osmotic shock*

Cells (3×10^8) were disrupted in 0.01 M- MgCl_2 as described in Table 6. Sucrose (1.5 M) was added immediately to a final concentration of 0.25 M and the suspension was rehomogenized rather gently. The subcellular fractions were separated as in Table 4.

Fraction	Total nucleic acid ($\mu\text{g. of P/10}^8$ cells)	DNA ($\mu\text{g. of P/10}^8$ cells)	Protein (mg./ 10^8 cells)
Nuclei + whole cells	250	228	6.6
Intermediate	4.2	1.9	0.13
Mitochondria	22.2	1.7	1.4
Microsomes	124	0.95	2.0
Cell sap	34.6	0.0	6.3

Under these circumstances nuclei swell, but do not burst, while whole cells are rapidly disrupted. This process was relatively independent of sucrose concentration and was observed to occur in both 0.1M- and 0.8M-sucrose solutions. Both Krebs II and Ehrlich ascites-tumour cells, but not isolated single rat-liver cells (Branster & Morton, 1957), behaved in this manner. The reversible swelling and shrinking of isolated nuclei when suspended in concentrated sucrose solutions followed by the addition of salts has been frequently observed (Anderson & Wilbur, 1952; Philpot & Stanier, 1956; Battin, 1959) but the rupture of ascites-tumour cells under similar circumstances has hitherto not been reported.

It is notorious that methods which give, in good yield, undamaged nuclei are usually less suitable for the isolation of other cell components (Allfrey, 1959), and this difficulty is accentuated when the cell membrane is unusually robust as it is in the Krebs cell. The success of the double-osmotic-shock method described here depends upon the exclusion of Ca^{2+} and Mg^{2+} ions during the swelling process and the addition of potassium chloride immediately after disruption.

Since the method gives quantitative recovery of RNA and DNA and clean separation between nuclei, mitochondria and microsomes, it has been used by us in further studies on the localization of the sites of metabolic change during the course of growth of encephalomyocarditis virus in Krebs II cells (Martin & Work, 1961). It has also been used extensively in the localization of the site of formation of infective RNA within these cells (Bellett & Burness, 1960). The method is not, however, completely satisfactory where the objective is the preparation of a microsome-cell sap mixture capable of protein biosynthesis, and for this purpose we have preferred mild osmotic shock in magnesium chloride and disruption with the Dounce homogenizer.

SUMMARY

1. Krebs II ascites-tumour cells can be ruptured by ultrasonics, by osmotic shock or by the high shear of a Dounce homogenizer.

2. Cells ruptured in a Ca^{2+} - and Mg^{2+} -free medium by a combination of osmotic shock and a low-shear homogenizer gave high yields of undamaged nuclei, mitochondria and microsomes.

3. Nuclei prepared by the osmotic method and separated from contaminating whole cells by a novel procedure, were free from succinoxidase and had low RNA:DNA ratio.

4. Microsomes prepared by osmotic-shock methods in the absence of Mg^{2+} ions were less active in incorporating radioactive valine than were microsomes prepared in the presence of Mg^{2+} ions by ultrasonics or by a high-shear homogenizer.

5. The Krebs II ascites-tumour cell is unusually robust and cannot be ruptured by methods which are adequate for other types of cell.

We wish to thank Mr Brian Rice for skilled assistance throughout this investigation.

REFERENCES

- Allen, E. H., Glassman, E. & Schweet, R. S. (1960). *J. biol. Chem.* **235**, 1061.
- Allfrey, V. G. (1959). In *The Cell*, vol. 1, p. 193. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.
- Allfrey, V. G., Mirsky, A. E. & Stern, H. (1955). *Advanc. Enzymol.* **16**, 411.
- Anderson, N. G. & Wilbur, K. M. (1952). *J. gen. Physiol.* **35**, 781.
- Battin, W. T. (1959). *Exp. Cell Res.* **17**, 59.
- Bellett, A. J. D. & Burness, A. T. H. (1960). *Biochem. J.* **77**, 17P.
- Branster, M. V. & Morton, R. K. (1957). *Nature, Lond.*, **180**, 1283.
- Dallam, R. D. (1954). *Fed. Proc.* **13**, 195.
- Davies, R. (1959). *Biochim. biophys. Acta*, **33**, 481.
- Dounce, A. L. (1955). In *The Nucleic Acids*, vol. 2, p. 93. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Hawtrey, A. O. & Silk, M. H. (1960). *Biochem. J.* **74**, 21.
- Klein, G. & Klein, E. (1951). *Cancer Res.* **11**, 466.
- Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.
- Lovelock, J. E. (1954). *Biochem. J.* **56**, 265.
- McComb, R. B. & Yushok, W. D. (1959). *Biochim. biophys. Acta*, **34**, 515.
- Martin, E. M., Malec, J., Sved, S. & Work, T. S. (1961). *Biochem. J.* **80**, 585.
- Martin, E. M. & Work, T. S. (1961). *Biochem. J.* (in the Press).
- Philpot, J. St L. & Stanier, J. E. (1956). *Biochem. J.* **63**, 214.
- Sauer, L. A., Martin, A. P. & Stotz, E. (1960). *Cancer Res.* **20**, 251.
- Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.