Incorporation of Radioactive Phosphorus into the Ribonucleic Acid of Subfractions Derived from Guinea-Pig-Liver Microsomes

By P. M. BHARGAVA, J. L. SIMKIN AND T. S. WORK
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 11 *July* 1957)

It is now generally accepted that there is a particularly rapid protein turnover in the microsome fraction of liver in various animals. We have already shown (Simkin & Work, 1957*a*) that the microsome fraction of guinea-pig liver can be separated by successive extractions with solutions of varying ionic strength and pH into several distinct subfractions, each containing proteins with different turnover rates. Two of these subfractions, *B* and *C*, appeared to be ribonucleoproteins, and after injection of [^{14}C]amino acids into normal guinea pigs the protein components of one of these two fractions (*C*) became labelled more rapidly than the other. Since a considerable amount of evidence has accumulated recently which suggests that ribonucleic acid (RNA) plays a special role in protein synthesis (Brachet, 1955), it seemed desirable to compare RNA turnover with protein turnover in the various subfractions from liver microsomes.

In the experiments described here, radioactive orthophosphate was injected into guinea pigs which were killed at appropriate time intervals. The microsome fraction was isolated and subfractionated as described earlier and the rate of incorporation of ^{32}P into the RNA of the different microsome subfractions was measured. It was found that in subfraction *C*, which had the highest rate of protein turnover, the rate of incorporation of ^{32}P into RNA was below that observed for any other microsomal subfraction.

EXPERIMENTAL

Estimation of ribose. The orcinol method of Mejbaum (1939) was used.

Spectrophotometric estimation of ribonucleic acid. The RNA was estimated by the method of Ogur & Rosen (1950); it was assumed that, under these conditions, RNA had an ϵ (P)$_{260}$ of 10 800. Optical density was measured with the Unicam SP. 500 spectrophotometer.

Estimation of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Animals. Guinea pigs (male and female) from the Institute stock were employed (body wt. 700–800 g.). The animals were starved overnight before injection, except in the 18 hr. experiment, in which they were starved only after injection.

Injection of ^{32}P. Inorganic phosphate ([^{32}P]orthophosphate) in isotonic saline (Radiochemical Centre, Amersham, Bucks) was injected intravenously. For each time point two animals were used, each being injected with 500 μc of ^{32}P (500 μg. of P).

Isolation of microsome fraction. The animals were killed after the appropriate time interval by a blow on the head. The livers were removed as rapidly as possible, chilled, minced, homogenized in 0·25 M-sucrose and the homogenate was centrifuged by the procedure of Simkin & Work (1957*a*) to isolate the microsome fraction. The temperature was maintained near to 0° throughout.

Fractionation of the microsome material. Fractionation was started in each case with the microsome pellet obtained from 36 g. of liver mince. The pellet was washed twice with glycerol (Simkin & Work, 1957*a*) and the microsome material was then suspended by homogenizing in about 10 ml. of 0·15 M-NaCl; 1 ml. samples were taken for (*a*) protein estimation and (*b*) isolation of RNA, and the remaining material was fractionated by the method of Simkin & Work (1957*b*) to give subfractions A_1, A_2, B_1 (referred to subsequently as *B*), B_2, *C*, *D* and *E* (about 12 ml. of extractant was used for each of the fractions A_1, *B*, *C*, *D* and *E* and 24 ml. for A_2 and B_2). Samples (1 ml.) were taken from each fraction for (*a*) protein estimation and (*b*) determination of ultraviolet-absorption spectrum, and the remaining material was used for the isolation of RNA. The RNA of fractions A_1, A_2 and B_2 was estimated only in one (18 hr.) experiment; at most, only a trace of RNA was detected, which is in accord with earlier observations (Simkin & Work, 1957*a*).

Isolation of nucleic acid from the whole microsome material and from the subfractions. Each fraction was mixed at 0° with 30% (w/v) trichloroacetic acid (TCA) to give a final TCA concentration of 5%. The precipitate was collected by centrifuging and washed once with 5% TCA at 0°. Samples of the combined TCA supernatants were used for estimation of ribose; this ribose is referred to as the 'TCA-soluble ribose'. The TCA-insoluble material was washed two or three times with 5% (w/v) NaCl in the cold, and then, to remove lipids, twice each with ethanol, acetone, ethanol–ether (3:1, v/v) and finally once with ether. In the 1 hr. experiment, ribose estimations were made on the combined NaCl washes and on the combined organic solvents' washes; the sum of these two values has been termed 'ribose in washes'. RNA was extracted from the fat-free residue by three successive 1 hr. extractions at 100°, 1 ml. of 10% (w/v) NaCl being used for each extraction. Since some RNA remained unextracted by this treatment, the protein residue was digested with 2 ml. of M-perchloric acid (PCA) overnight at room temperature (cf. Ogur & Rosen, 1950) and ribose was estimated in the digest; this is referred to as the 'PCA-soluble ribose'. The 10% NaCl extracts were made up to 3·5 ml. and ribose was estimated on 0·2 ml. samples after perchloric acid digestion; this is referred to as the '10% NaCl soluble ribose'. As a check on

the above method, RNA was also estimated spectrophotometrically on 0·2 ml. samples of the NaCl extract from whole microsomes (*M*) and from fraction *B* (6 hr. experiment); the values were found to be 90 and 96% respectively of those obtained by the orcinol method. Ribonucleic acid was precipitated from the remainder of the NaCl extracts by addition of 7 ml. of ethanol, and was collected after leaving for 16 hr. at −4°. The precipitate was washed once each with ethanol and ether at 0°. In the 1, 6 and 18 hr. experiments, the combined ethanol and ether supernatants were evaporated to dryness and ribose estimations were made on the aqueous extract of the residue; this has been designated the 'ethanol-soluble ribose'.

Determination of radioactivity. The samples of RNA were dissolved in water, plated on aluminium planchets and the radioactivity of the samples was measured in a thin-window Geiger counter; in the 18 hr. experiment only a portion of the aqueous solution was plated. The amount of RNA on the planchet was obtained (except in the 30 min. experiment) by subtracting the amount of RNA left unprecipitated by ethanol from the amount of RNA in 10% NaCl extracts (method I). For conversion of ribose values into RNA values a factor of 4·38 was used; this factor is based on the nucleotide ratios of rabbit-liver microsomes (with adenylic acid as 1·0; guanylic acid = 1·58; cytidylic acid = 1·54; uridylic acid = 1·03) reported by Crosbie, Smellie & Davidson (1953). In the 30 min. and 1 hr. experiments, after counting, the sample was washed off the planchet with 0·01 N-NaOH (it was not found possible to remove all the radioactivity from the planchets by water), and RNA was estimated spectrophotometrically. (Method II.) In the 1 hr. experiment where both methods, I and II, were used for the subfractions *B* and *C*, the quantities of RNA as determined by the former method were 98 and 94% respectively of the values obtained by the latter method.

Separation of individual nucleotides by electrophoresis. The method used was essentially that of Davidson & Smellie (1952*a*). Since the amount of nucleic acid available from each fraction was small, it was found necessary to dilute it with RNA of known composition before hydrolysis. The dried sample of nucleic acid from fraction *B*, *C* or *D* (18 hr. experiment) was dissolved in 0·5 ml. of a stock solution of yeast RNA (nucleotide composition with adenylic acid as 1·0: guanylic acid = 1·17, cytidylic acid = 0·70; uridylic acid = 0·94) in water (568 μg. of RNA or 55 μg. of RNA-P/ml.); 0·1 ml. of the mixture was used for determination of specific activity as already described, and the rest was hydrolysed with 0·4 ml. of 0·6 N-KOH for 22 hr. at 37°. The pH of the solution was adjusted to 4·0 by the addition of the calculated quantity of perchloric acid and the $KClO_4$ removed. A measured volume (0·2–0·4 ml.) of this solution was submitted to electrophoresis for 8 hr. on Whatman no. 3 MM paper (57 cm. × 33 cm.; previously washed free of ultraviolet-absorbing material by elution with 0·2 N-HCl in a chromatography chamber for 48 hr.), an apparatus based on that of Kunkel & Tiselius (1951) being used. Citrate buffer of pH 3·5 was used, with a potential difference of 550 v and a gradient of 10 v/cm. (cf. Davidson & Smellie, 1952*a*). Nucleotides were located by ultraviolet photography; the spots were eluted with 0·02 N-HCl (5 ml.) and the nucleotides estimated by measurement of optical density at 260 mμ (the washed-paper blank, extracted in the same way, gave $E^{260}_{1\,cm.} = 0·0033$/cm.^{2} of the paper). To check the identity of each nucleotide, absorbance ratios were determined at the following wavelengths, 250/260, 270/260 and 290/260 mμ; these agreed within experimental error with those given by Beaven, Holiday & Johnson (1955). The material eluted from each spot was then plated on an aluminium planchet and the radioactivity was measured. Good separation of nucleotides was obtained with 0·3–0·4 μg. of nucleotide P, and a migration time of 8 hr. Relative mobilities were (taking uridine monophosphate = 1·0): cytidine monophosphate 0·07; adenosine monophosphate 0·23; guanosine monophosphate 0·78. Davidson & Smellie (1952*a*) give somewhat different mobility values, but they used the Durrum (1950) open-strip type of electrophoresis apparatus.

Incorporation of ^{32}P *into microsomal ribonucleic acid in a cell-free system.* The livers from two guinea pigs were minced and homogenized in 0·25 M-sucrose under the conditions described by Simkin & Work (1957*b*). The nuclei and mitochondria were removed and the microsome–cell-sap fraction (7·5 ml., equivalent to 3 g. of liver) was incubated with the mixture of salts and 3-phosphoglycerate used earlier in experiments on amino acid incorporation (Simkin & Work, 1957*b*), and with either 25 μc or 125 μc of ^{32}P. Incubation was carried out at 37° in an atmosphere of $N_2 + CO_2$ (95:5). At appropriate times, the reaction was stopped by the addition of 15 ml. of an ice-cold solution containing sodium phosphate buffer (mM, pH 7·4) and sucrose (0·25 M). The cold suspension was centrifuged at $g_{av.}$ 78 000 as described by Simkin & Work (1957*b*) and RNA was isolated from the unwashed microsome pellet by the procedure described earlier. In this experiment the microsome material was not separated into individual subfractions.

RESULTS

Incorporation of ^{32}P *into the ribonucleic acid of liver microsomes* in vivo

The rate of incorporation of ^{32}P into the RNA of liver-microsome material was rather low and it was not possible to follow incorporation over the same time scale as was used earlier in studying protein turnover (Simkin & Work, 1957*a*). As can be seen from Table 1, the radioactivity of the RNA from the liver-microsome subfractions, isolated as

Table 1. *Specific radioactivity of ribonucleic acid isolated from microsomes of guinea-pig liver and from subfractions derived therefrom*

Animals were killed at various time intervals after the injection of [^{32}P]phosphate. *M* represents the whole microsome fraction; *B*, *C*, *D* and *E* are the subfractions derived from *M*. For method of fractionation see text. Specific activity is expressed as counts/min./mg. of RNA.

Time	Specific activity of RNA				
(hr.)	*M*	*B*	*C*	*D*	*E*
0·5	31	19	8	106	250
1·0	64	40	25	168	—
6·0	2240	1680	1750	2860	3580
18·0	—	4630	3900	5200	7210

described in the Experimental section, increased with time, but with some initial lag. The initial rate of uptake into the nucleoprotein fraction *C* was notably slower than that observed for other subfractions.

It seemed likely that the material extracted into 10 % NaCl and precipitated by ethanol was entirely RNA, particularly since estimation by ultraviolet spectroscopy after perchloric acid digestion gave values within 6 % of those obtained with the ribose method, but since with small quantities of RNA slight contamination with other radioactive phosphorus compounds might vitiate the results (cf. Davidson, Frazer & Hutchison, 1951; Davidson & Smellie, 1952*b*), the specific activity of individual nucleotides obtained by hydrolysis of RNA was also determined. This could not be done for the materials isolated from animals killed at short time intervals after injection, since the combination of low specific activity and small quantity of RNA would have reduced the specific-activity values for individual nucleotides to insignificant levels.

Satisfactory results were obtained with the RNA from subfractions of microsomes in the 18 hr. experiment. In this experiment, the RNA precipitated by ethanol after extraction into 10 % NaCl (subfractions *B*, *C* and *D*) was diluted with yeast RNA and hydrolysed as described in the Experimental section. The specific radioactivities of the individual nucleotides, separated by ionophoresis on paper, are given in Table 2. The recoveries of nucleotide from fractions *B*, *C* and *D* were 72, 79 and 86 % respectively, which correspond well with the radioactivity recoveries of 70, 83 and 94 % (the recoveries are not quantitative because the area of paper occupied by nucleotide is not always clearly enough defined to permit exact delineation of the spot for elution). The results (Table 2) showed that the RNA isolated by extraction with 10 % NaCl and precipitation with ethanol was free from radioactive contaminants and that subfraction *D* did indeed become more rapidly labelled than either subfraction *B* or *C*. Subfraction *E* did not contain enough RNA to permit separation of individual nucleotides, but since no contamination was detected in fraction *D*, it seems reasonable to assume that the RNA from fraction *E* was also free of radioactive contaminants.

Although RNA free from phosphorus contaminants could be isolated from the lipid-free microsome subfractions by extraction with hot NaCl soln. followed by precipitation with ethanol, there were considerable losses during isolation, particularly for subfractions *D* and *E*, due either to incomplete extraction or to incomplete precipitation. In these subfractions, a considerable amount of ribose-containing material, presumably obtained by degradation of RNA, was also found in the TCA

Table 2. *Distribution of radioactivity between different nucleotides obtained by hydrolysis of ribonucleic acid labelled with* ^{32}P

RNA was isolated from the subfractions *B*, *C* and *D* (obtained from the 18 hr. expt.) as described in the text. It was mixed with carrier yeast RNA, hydrolysed with alkali and the individual nucleotides were separated by paper electrophoresis as described in the text. Specific activity is expressed as counts/min./μmole of P.

Fraction	Specific activity of RNA	Specific activity of nucleotides*				Recovery of nucleotides† (%)	Recovery of radioactivity† (%)
		Ad	Gu	Cy	Ur		
B	1495	1535	1615	1290	1240	72	70
C	1268	1900	1670	965	1160	79	83
D	1685	2240	2005	1565	1510	86	94

* Ad, adenylic acid; Gu, guanylic acid; Cy, cytidylic acid; Ur, uridylic acid.

† These figures are the sum of the values for the four nucleotides and are expressed as percent of the calculated total nucleotide material or total radioactivity taken for electrophoretic separation.

Table 3. *Ribose content of different subfractions obtained from the microsome material of guinea-pig liver*

For method of fractionation of microsomes and of obtaining the various ribose-containing fractions from each microsomal subfraction, see text. All values are given as the mean of the four experiments, and are followed by the standard deviation. In each experiment 36 g. of liver mince was used.

Microsomal subfraction	10 % NaCl soluble ribose (μg.)	TCA-soluble ribose (μg.)	Ribose in washes* (μg.)	PCA-soluble ribose (μg.)	Ethanol-soluble ribose (μg.)
B	533 ± 75	24 ± 3	23	91 ± 13	27 ± 8
C	191 ± 29	29 ± 3	9	32 ± 8	15 ± 5
D	43 ± 10	23 ± 5	7	41 ± 9	14 ± 8
E	16 ± 4	9 ± 2	9	12 ± 5	11 ± 3
$A_1 + A_2 + B_2$*	75	—	—	15	41

* Estimated in one experiment only.

supernatant and subsequent washes after the initial precipitation. The yield of RNA in the 10% NaCl extracts, and the various losses which occurred during isolation and purification of the RNA, are listed in Table 3. It is obvious that losses increased as the amount of RNA in the fraction decreased. Such losses seem unavoidable if reasonably pure samples of RNA are to be obtained. There were also quite substantial variations in the amounts of RNA obtained in different experiments as shown by the standard deviation values given in Table 3. This seemed to be due to normal biological variations which might be expected in a cell component such as RNA, which fluctuates with the nutritional state of the animal (Wikramanayake, Heagy & Munro, 1953). In the experiments reported here, subfraction *B* had the highest RNA content and the highest RNA: protein ratio (the average RNA: protein ratios for fractions *B*, *C*, *D* and *E* were 0·56, 0·32, 0·048 and 0·0088 respectively). In earlier experiments, the RNA: protein ratio for fraction *B* was sometimes lower than that for fraction *C* (Simkin & Work, 1957*a*). This variability does not appear to affect the pattern of protein labelling in the liver-microsome subfractions (Simkin & Work, 1957*b*).

Incorporation of ^{32}P *into microsomal ribonucleic acid in a cell-free system*

When a microsome–cell-sap preparation was incubated with radioactive phosphate and an energy source (3-phosphoglycerate), there was significant incorporation of ^{32}P into the RNA of the microsome material. The results of experiments with two different levels of ^{32}P are given in Table 4. There was an increase in the specific radio-activity of microsomal RNA for at least 30 min., and this was accompanied by a steady loss of RNA from the microsome material as reported by Simkin & Work (1957*b*).

Since under similar conditions to those used in this investigation, uptake of radioactive amino acids ceases rather abruptly after about 20 min. incubation, it seemed unlikely that the two processes, amino acid incorporation and ^{32}P incorporation, were directly related and further experiments were not performed.

Table 4. *Specific radioactivity of ribonucleic acid from guinea-pig-liver microsomes incubated* in vitro *for different time periods*

The microsome–cell-sap preparation was incubated under conditions described in the text. In Expts. 1 and 2, the amount of radioactivity in the incubation mixture for each time point was 25 and 125 μc of ^{32}P respectively.

Time (min.)	Specific activity (counts/min./mg. of RNA)	
	Expt. 1	Expt. 2
0	21	86
15	46	187
30	101	392

DISCUSSION

Most cells which are capable of rapid protein synthesis are rich in ribonucleic acid, and, usually, protein synthesis and RNA synthesis seem to run in parallel during growth (Brachet, 1955). It is known also that exposure to ribonuclease frequently destroys the capacity of cells to synthesize protein (Brachet, 1955), and that removal of RNA from subcellular particles, either by exposure to ribonuclease or by extraction with sodium chloride, inhibits uptake of radioactive amino acids into proteins of these particles (Allfrey, Daly & Mirsky, 1953; Gale & Folkes, 1955; Marmur, Nisman & Hirsch, 1955). Therefore when we found (Simkin & Work, 1957*a*) that two ribonucleoprotein fractions could be obtained from guinea-pig-liver microsomes which differed considerably in their rate of protein turnover, it became a matter of considerable interest to determine the rate of RNA turnover in the same two fractions.

There is, in our results, no suggestion that RNA turnover runs parallel to protein turnover. Indeed, assuming that rates of uptake of radioactive amino acid and of radioactive phosphorus can be regarded as indices of the rates of protein and nucleic acid synthesis, it appears more likely that there is an inverse relationship between the rate of protein turnover and that of RNA. All experiments involving a single injection of isotope are complicated by rapid changes in radioactivity of precursor pools and by the possibility of intracellular-permeability barriers (cf. Reiner, 1953; Siminovitch & Graham, 1956), but since, in the present investigation, the ribonucleoprotein fractions are derived from microsome material, such difficulties are unlikely to vitiate results.

There is, in the literature, additional evidence consistent with the assumption of an inverse relationship between RNA turnover and protein turnover. Thus Hultin (1955), who first showed in 1950 that protein turnover is particularly rapid in liver microsomes, has emphasized that the RNA of soluble nucleoproteins of the cell sap has a higher rate of turnover than the microsomal RNA. Shigeura & Chargaff (1957) have recently fractionated microsomal material by a different method from that employed by us and have also found that a ribonucleoprotein fraction with a high protein-turnover rate had a particularly low rate of turnover of RNA. Smellie & Davidson (1956), working with several different ^{14}C-labelled precursors, have confirmed earlier work showing that cytoplasmic

RNA has a lower turnover rate than nuclear RNA, whereas for proteins the reverse is true. The results of recent elegant experiments of Clark, Naismith & Munro (1957), on the effect of protein starvation and protein feeding upon the rate of labelling of rat-liver RNA, point in the same direction. They found that RNA was stabilized by conditions favouring protein synthesis. It is thus clear that the results of the present investigation are in line with much other evidence obtained with mammalian cells. In each case, however, it is the rate of turnover of undefined trichloroacetic acid-precipitable protein which is being measured and where, as in micro-organisms, it has been possible to follow synthesis of a single induced enzyme, very different results have been obtained. Thus Pardee (1955), Gale (1956) and Spiegelman (1956) have all found that induced-enzyme formation was necessarily accompanied by synthesis of RNA. It is possible that simultaneous synthesis of RNA and protein is required only where new protein structures (e.g. induced enzymes) are being formed. In other cases of protein synthesis, as for example in the phenomenon of balanced synthesis and breakdown (protein turnover) in mature non-dividing mammalian cells, there may well exist an inverse relationship between the syntheses of protein and RNA. A precise explanation of this apparent contradiction may have to await elucidation of the exact role of RNA in protein synthesis.

SUMMARY

1. Guinea pigs were killed at various time intervals after intravenous injection of [^{32}P]phosphate. The liver-microsome fraction was subfractionated by the method of Simkin & Work (1957*b*). The ribonucleic acid of the various subfractions was isolated and the specific radioactivity of the nucleic acid from each subfraction was determined.

2. In one experiment, the ribonucleic acid isolated from three subfractions was hydrolysed to mononucleotides and their specific radioactivity was determined. On the basis of the results it was concluded that the ribonucleic acid was free from other ^{32}P-containing contaminants.

3. Incorporation of ^{32}P into the ribonucleic acid of the various microsome subfractions occurred at different rates. The ribonucleoprotein subfraction *C* had the lowest rate of ribonucleic acid turnover, although it has the highest rate of protein turnover.

4. These results are discussed in relation to other work on ribonucleic acid turnover during protein synthesis.

We are grateful to Dr D. C. Burke for a supply of yeast RNA of known nucleotide composition. One of the authors (P.M.B.) wishes to thank the Wellcome Trust for a Wellcome Research Fellowship which made this work possible.

REFERENCES

Allfrey, V. G., Daly, M. M. & Mirsky, A. E. (1953). *J. gen. Physiol.* **37**, 157.
Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press.
Brachet, J. (1955). *The Nucleic Acids*, vol. 2, p. 475. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press.
Clark, C. M., Naismith, D. J. & Munro, H. N. (1957). *Biochim. biophys. Acta*, **23**, 587.
Crosbie, G. W., Smellie, R. M. S. & Davidson, J. N. (1953). *Biochem. J.* **54**, 287.
Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1951). *Biochem. J.* **49**, 311.
Davidson, J. N. & Smellie, R. M. S. (1952*a*). *Biochem. J.* **52**, 594.
Davidson, J. N. & Smellie, R. M. S. (1952*b*). *Biochem. J.* **52**, 599.
Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
Gale, E. F. (1956). *Henry Ford Hospital Symposium, Enzymes: Units of Biological Structure and Function*, p. 49. New York: Academic Press.
Gale, E. F. & Folkes, J. P. (1955). *Biochem. J.* **59**, 661, 675.
Hultin, T. (1955). *Exp. Cell. Res. Suppl.* **3**, 210.
Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Marmur, J., Nisman, B. & Hirsch, M. L. (1955). *C.R. Acad. Sci., Paris*, **240**, 2025.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Pardee, A. B. (1955). *J. Bact.* **69**, 233.
Reiner, J. M. (1953). *Arch. Biochem. Biophys.* **46**, 53.
Shigeura, H. T. & Chargaff, E. (1957). *Biochim. biophys. Acta*, **24**, 451.
Siminovitch, L. & Graham, A. F. (1956). *J. Histochem. Cytochem.* **4**, 508.
Simkin, J. L. & Work, T. S. (1957*a*). *Biochem. J.* **65**, 307.
Simkin, J. L. & Work, T. S. (1957*b*). *Biochem. J.* **67**, 617.
Smellie, R. M. S. & Davidson, J. N. (1956). *Experientia*, **12**, 422.
Spiegelman, S. (1956). *Henry Ford Hospital Symposium, Enzymes: Units of Biological Structure and Function*, p. 67. New York: Academic Press.
Wikramanayake, T. W., Heagy, F. C. & Munro, H. N. (1953). *Biochim. biophys. Acta*, **11**, 566.