For TDP Westenbrink, Goudsmith & Veldman (1941) reported ($\mu\text{g./g.}$): brain, 3.0; heart, 6.0; kidney, 5.5; liver, 7.0–13.0; and Boffi, Bucci & Lucarelli (1958) for the same tissues: 2.8; 6.6; 4.9; 6.7. In all our determinations, small amounts of TMP and TTP were always found in animal tissues along with thiamine and TDP. Their biochemical significance has still to be discovered. TTP was found in liver by Rossi-Fanelli *et al.* (1952) and in liver, kidney and brain by Greiling & Kiesow (1958). Our observations showed its presence in heart too, but the richest organ analysed was the liver (0.84 $\mu\text{g./g.}$), followed by the heart (0.59 $\mu\text{g./g.}$), kidney (0.27 $\mu\text{g./g.}$) and brain (0.19 $\mu\text{g./g.}$). The most abundant thiamine compound in rat tissue is TDP, which represents an average of about 80 % of the total thiamine present.

SUMMARY

1. A new analytical procedure for the determination of thiamine and its mono-, di- and triphosphoric esters in rat tissue is described.

2. The method allows a mean thiamine recovery of 95 %, and good reproducibility.

3. In all the rat tissues studied, thiamine diphosphate was the most abundant thiamine phosphoric ester, but small amounts of thiamine tri- and mono-phosphate were also found.

REFERENCES

- Association of Vitamin Chemists (1951). *Methods of Vitamin Assay*, 2nd ed., p. 111. New York: Interscience Publishers Inc.
- Bartley, W. (1954). *Biochem. J.* **56**, 379.
- Bernabei, O. & Wildemann, L. (1959). *Naturwissenschaften*, **46**, 229.
- Bessey, O. A., Lowry, O. H. & Davis, E. B. (1952). *J. biol. Chem.* **195**, 453.
- Boffi, V., Bucci, E. & Lucarelli, A. (1958). *Int. Z. Vitaminforsch.* **29**, 1.
- de Giuseppe, L. & Rindi, G. (1958). *J. Chromat.* **1**, 545.
- Dreyfus, P. M. (1959). *J. Neurochem.* **4**, 183.
- Greiling, H. & Kiesow, L. (1958). *Z. Naturf.* **13B**, 251.
- Gurtner, H. P. (1957). *Helv. physiol. acta*, **15**, C66.
- Kaziro, Y. (1957). *J. Biochem., Tokyo*, **44**, 827.
- Kiessling, H. K. & Lindahl, G. (1953). *Ark. Kemi*, **6**, 271.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Rossi-Fanelli, A., Siliprandi, N. & Fasella, P. (1952). *Science*, **116**, 711.
- Siliprandi, D. & Siliprandi, N. (1954). *Biochim. biophys. Acta*, **14**, 52.
- Spadoni, M. A. & Tecce, G. (1950). *Quad. Nutr.* **11**, 26.
- Vannatta, E. E. & Harris, L. E. (1959). *J. Amer. pharm. Ass.* **48**, 30.
- Vescia, A. & Testi, G. (1958). *G. Biochim.* **7**, 115.
- Westenbrink, H. G. K., Goudsmith, J. & Veldman, H. (1941). *Vitam. & Horm.* **1**, 15.
- Westenbrink, H. G. K. & Steyn-Parvé, E. P. (1949). *Int. Z. Vitaminforsch.* **21**, 461.

Biochem. J. (1961) **78**, 606

The Distribution of Free mesoInositol in Mammalian Tissues, Including some Observations on the Lactating Rat

By R. M. C. DAWSON AND N. FREINKEL*

Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, and Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, Mass., U.S.A.

(Received 10 October 1960)

Since Woolley (1940) first demonstrated that mesoinositol was an essential dietary factor for the growth of the mouse, there have been several studies on the distribution of total tissue inositol (e.g. Taylor, Pollack & Williams, 1942) and lipid inositol (e.g. Taylor & McKibbin, 1953) in mammals. Surprisingly little attention appears to have been paid to the distribution of free inositol in the tissues, although Woolley (1942) found that 60 % of the inositol present in the normal mouse carcass was in the free form, and Platt & Glock (1943) obtained

similar results with the rat. Meyer (1946) isolated free crystalline inositol from commercial desiccated thyroid powder, and more recently Kojima & Kusakabe (1954) have obtained it from the brain and kidneys of the pig. Mann (1954) has isolated large amounts of inositol from the seminal vesicular secretion of the boar.

The present paper presents results of assays for free mesoinositol in the tissues of the rat, guinea pig and rabbit by using the specific microbiological-assay procedure of Campling & Nixon (1954), which can estimate free inositol in the presence of esterified inositol. It shows that the concentration

* Investigator, Howard Hughes Medical Institute.

of free inositol varies considerably from organ to organ, the highest being found in the pituitary and thyroid glands. However, in all the tissues its concentration is appreciably higher than the corresponding value for the plasma, which indicates that there is a concentration gradient for free inositol between cellular and extracellular fluids. In the rat mammary gland the concentration of free inositol was found to be much higher when the animal was lactating. Because of the possible importance of inositol in reproduction and lactation (Hamilton & Hogan, 1944; Schweigert, 1948), the present studies were extended to include an investigation of the free inositol present in the mammary gland and certain other tissues of the rat at various times before parturition and during lactation.

EXPERIMENTAL

Animals and preparation of tissue extracts. The rats used were male and female adult Sprague-Dawley descendants (Charles River Breeding Laboratories, Brookline, Mass.) fed *ad lib.* on a diet of Purina chow pellets (inositol content 0.065% free, 0.114% total). They were either killed by a blow on the head or deeply anaesthetized with ether. The abdomen was quickly opened, a blood sample taken from the abdominal aorta, and the tissues were dissected, weighed on a torsion balance and immediately frozen on solid CO₂. Guinea pigs were anaesthetized with ether and rabbits killed by air embolism or cervical dislocation. Tissues from all animals were removed within 5–10 min. after induction of anaesthesia or death of the animals. Tissues were excised in random sequence to minimize systematic error due to catabolism of esterified inositol.

Four methods of protein precipitation were tried. Trichloroacetic acid followed by ether extraction of the aqueous extract was satisfactory except when the inositol content of the tissue was low. This necessitated using more tissue extract, and a precipitate often developed on autoclaving with the yeast growth medium. A similar trouble occurred on heat denaturation at pH 5, and protein removal with acid ZnSO₄-NaOH sometimes gave irregular growth when inositol standards were used. A satisfactory procedure appeared to be HClO₄ precipitation followed by the removal of perchlorate ions as KClO₄.

The tissue (0.6 g. or less) was homogenized in a Potter-Elvehjem homogenizer with 12 ml. of ice-cold 5% perchloric acid solution, and 1 ml. samples of plasma or blood were treated directly with 11 ml. of 5% HClO₄. After 5 min. the suspension was centrifuged, and the clear or slightly turbid supernatant filtered through a retentive paper (Whatman no. 42). A 7 ml. sample of the filtrate was added to a stoppered graduated centrifuge tube, and 5N-KOH (approx. 1.4 ml.) added until the pH was about 5 to indicator paper. The volume was made up to 10 ml., and the tube cooled in ice for 30 min. and then centrifuged in the cold to remove the precipitated KClO₄.

Inositol assay. Inositol was determined in an appropriate volume of the HClO₄ extract by the method of Campling & Nixon (1954). The *Kloeckera brevis* yeast used (National Collection of Yeast Cultures, no. 328) shows a highly specific response for free inositol (Hartree, 1957),

and its growth is not influenced by the presence of choline (Lovern & Olley, 1953). The growth medium in later experiments was buffered by the addition of 10% of its volume of 0.2M-sodium acetate-acetic acid buffer, pH 5.2. After growth for 3 days at 25° with shaking once daily, the turbidities were read in a Hilger and Watts Spekker photoelectric absorptiometer with a Kodak no. 8 dark-red filter, or a Unicam or Beckman DU spectrophotometer at 660 mμ. All estimations were performed in duplicate and these usually agreed within about 5%. Standard curves (0.5–5 μg. of inositol) were set up for each set of estimations and were virtually straight lines passing through the origin. Above 5 μg. of inositol a flattening of the dose-response curve was observed. Therefore all specimens were appropriately diluted for analysis. There was no evidence that any of the tissue extracts possessed a toxic factor which inhibited the yeast growth: indeed, low dilutions of the extracts tended to give slightly lower recoveries of inositol (10%). This might be due to adsorption of inositol on the glass or possibly some factor which enhances yeast growth in the tissue extracts.

Chromatographic identification of free inositol in thyroid. The thyroid glands from two guinea pigs were homogenized in 20 ml. of 60% ethanol. The suspension was centrifuged and the supernatant was shaken with 60 ml. of chloroform. The emulsion was centrifuged and the upper aqueous layer was shaken with 3 vol. of butan-2-ol. The lower layer which separated was used for chromatography. Samples of 0.1 ml. were chromatographed on paper in the solvents: ethanol-aq. NH₃ soln. (sp.gr. 0.880)-water (6:3:1, by vol.) and propan-2-ol-acetic acid-water (3:1:1, by vol.).

Inositol was detected by the acetone-AgNO₃ and ethanolic NaOH reagents described by Trevelyan, Procter & Harrison (1950). By using various quantities of pure inositol as markers, a rough quantitative measure of the amount of inositol present in the tissue could be obtained by visual comparison of the spots.

RESULTS

Free inositol in the tissues of the normal rat. In Table 1 are presented the concentrations of free inositol in a number of tissues of the adult rat. The organs were taken from the carcass in differing orders for each animal but no evidence was obtained that the concentration of inositol changed significantly in the immediate post-mortem period. This agrees with similar results that were obtained for thyroid tissue where the gland was either frozen immediately after removal or 4 min. afterwards (Freinkel, Dawson, Ingbar & White, 1959).

The highest concentrations of free inositol were found in the thyroid and pituitary glands, although appreciable concentrations were also found in the kidney cortex and the reproductive organs. In the thyroid and pituitary glands the presence of large amounts of free inositol was confirmed by paper chromatography of aqueous-ethanol extracts, which yielded inositol spots of approximately the size and density expected from the values obtained by yeast bioassay. The mean concentrations of free inositol in liver, heart and kidney

Table 1. *Distribution of free inositol in the tissues of various species*
Values are given as mg. of inositol/100 g. of tissue or 100 ml. of plasma and blood.

	Rat			Guinea pig			Rabbit		
	No. of animals	Range	Mean $\pm$ s.d.	No. of animals	Range	Mean $\pm$ s.d.	No. of animals	Range	Mean $\pm$ s.d.
Plasma	31	0.22-1.80	1.14 $\pm$ 0.45	4	0.93-3.7	1.70 $\pm$ 1.32	6	0.55-1.20	0.77 $\pm$ 0.23
Whole blood	5	0.75-1.61	1.25 $\pm$ 0.35	5	0.75-2.0	1.19 $\pm$ 0.50	2	0.58-0.75	0.68
Liver	30	6.0-24.5	12.1 $\pm$ 3.4	5	0.19-0	9.5 $\pm$ 7.6	5	14-29	23 $\pm$ 6
Lung	6	5.6-33.1	17.1 $\pm$ 9.2	4	5-76	38	4	63-140	87 $\pm$ 35
Heart	6	4.9-8.5	7.1 $\pm$ 1.3	4	3-25	15 $\pm$ 10	5	17-73	33 $\pm$ 23
Kidney cortex	28	30-136	61 $\pm$ 26	5	82-132	107 $\pm$ 18	5	71-116	87 $\pm$ 21
Testes	9	14-33	28 $\pm$ 7	2	218-258	238	3	62-113	80
Ovary	8	8-56	32 $\pm$ 16	2	182-196	189	—	—	—
Thyroid	24	57-255	121 $\pm$ 44	8	53-550	324 $\pm$ 157	7	126-350	227 $\pm$ 81
Pituitary	15	80-120	96 $\pm$ 13	—	—	—	3	199-714	424
Adrenal cortex	14	6-28	16 $\pm$ 6	5	148-289	206 $\pm$ 53	6	39-67	51 $\pm$ 10
Salivary gland	7	2-34	19 $\pm$ 11	5	51-99	69 $\pm$ 21	1	—	85
Brain (cerebral cortex)	—	—	—	—	—	—	5	201-263	241 $\pm$ 24
Spleen	6	6-40	22 $\pm$ 11	5	84-125	105 $\pm$ 19	3	121-153	134
Diaphragm	3	5.2-7.7	6.2	—	—	—	—	—	—
Pancreas	1	—	12	5	48-73	59 $\pm$ 9	5	46-97	68 $\pm$ 20
Adipose tissue	2	2.1-4.2	3.1	—	—	—	—	—	—
Eye (lens)	—	—	—	—	—	—	4	16-206	100

are considerably below those found by Platt & Glock (1943), who used a chemical method of estimation which is likely to be relatively non-specific compared with the microbiological assay. In the pituitary gland more than 90 % of the free inositol is selectively located in the anterior portion, although the minute amounts of posterior pituitary which were available for analysis made the values obtained only very approximate. In additional experiments rat thyroid and pituitary glands were homogenized in the sucrose medium of Hecht, Stephenson & Zamecnik (1959). After centrifuging at 77 000-105 000 *g* for 1 hr. at 4° the free inositol was found to be exclusively located in the supernatant fraction. The amounts of inositol recovered were equivalent to those found after perchloric acid extraction of glands.

The mean concentration of free inositol in rat plasma was more than twice that found in man (Sonne & Sobotka, 1947; Campling & Nixon, 1954; Freinkel *et al.* 1959), but nevertheless in every organ the concentration of free inositol was appreciably above that in the plasma. Mean tissue-plasma gradients were as high as 106 and 84 for the thyroid and pituitary respectively. From the values obtained for whole blood it can be deduced that no appreciable concentration gradient of free inositol exists between the cellular components of the blood and the plasma, which is similar to the conclusions reached by Campling & Nixon (1954) for human blood.

Free inositol in the tissues of the lactating rat. It was clear from initial analyses that during lactation the concentration of inositol in the rat mammary gland was appreciably higher than normal. The investigation was therefore extended to include

Fig. 1. Inositol levels in the mammary glands (●) and livers (○) of female adult rats *ante partum* and *post partum*. A shows the range of values in non-pregnant animals.

analyses of the mammary glands and other tissues of rats at various times during the immediate *ante partum* and *post partum* periods. In these animals the mammary glands were found on dissection to be clearly defined anatomical structures, in contrast to the ill-defined avascular rudimentary glands of non-pregnant animals.

Fig. 1 shows that the inositol content of the mammary gland increases markedly in the *post partum* period, corresponding to the onset of lactation. No similar increase was seen either in the liver or kidney cortex of these animals; in fact in the latter there was a small but significant fall in the inositol concentration from 74 mg./100 g. *ante partum* to 52 mg./100 g. *post partum* ($P = 0.01$). Plasma levels remained more or less in the normal range in all the animals except in three which were killed 3 days *post partum*, in which the inositol concentrations were about half the normal value (0.36, 0.62, 0.38 mg./100 ml. of plasma).

Free inositol in tissues of the guinea pig and rabbit. Table 1 shows the distribution of free inositol in the tissues of the guinea pig and rabbit. In both species the concentrations of free inositol in the tissues appear to be in general higher than those in the rat with the exception of rabbit plasma and guinea-pig liver. This difference is especially marked in the adrenal gland where the inositol content is about ten times as high in the guinea pig as in the rat. Similarly, the ovary, testes, salivary gland and spleen inositol concentrations are many times the equivalent ones found in the rat.

The thyroid gland, as in the rat, contained inositol in very high concentrations, although in the rabbit the mean level was surpassed by that of the pituitary. Again the thyroid-plasma concentration gradients were very high, the mean gradients amounting to 295 in the rabbit and 195 in the guinea pig. The high concentration of inositol in the rabbit cerebral cortex must be viewed with reservations, owing to the presence in nervous tissue of enzymes which rapidly liberate free inositol from the inositol-containing phospholipids (Sloane-Stanley, 1953; Rodnight, 1956).

DISCUSSION

The high concentrations of free inositol which occur in mammalian tissues, especially in the endocrine glands, was a rather unexpected finding of the present survey. In the thyroid and pituitary glands, for example, it must exist as one of the major water-soluble metabolites of the tissue and account for a very considerable part of the dry weight of the glands (1–2% in guinea pig and rabbit).

The method of assay used for free inositol relies on the premises that no acid-labile precursor of

inositol is broken down during the perchloric acid protein precipitation and that the *K. brevis* yeast used in the assay only grows in response to free inositol. The correctness of these assumptions has been demonstrated at least for the thyroid gland by experiments in which (1) the gland was merely homogenized in neutral sucrose media, and normal levels of free inositol were obtained on bioassay, and (2) the gland was extracted with aqueous 60% ethanol, and free inositol was observed on paper chromatography of the extracts in amounts equivalent to those found by bioassay.

It is apparent that a very considerable concentration gradient of free inositol exists between the tissues and plasma. In the rat thyroid and pituitary, for example, the average concentration of inositol in the glands is more than 80 times that in the plasma, and even higher concentration gradients are found with these glands in the rabbit and guinea pig. In the rat it has been shown that the inositol in the thyroid and pituitary is present in the cytoplasmic supernatant fraction obtained on centrifuging sucrose homogenates. This means that a considerable permeability barrier to intracellular free inositol must exist in the cell walls.

The high concentration of inositol in tissue cells compared with the plasma can be contrasted with the hexoses, e.g. glucose or fructose, for which a concentration gradient between tissues and plasma has never been demonstrated. Recently, however, accumulation of soluble carbohydrate derivatives has been observed in certain bacteria (Rickenberg, Cohen, Buttin & Monod, 1956).

The high concentrations of inositol in the tissues raises the question of whether they are attained by active transport from the plasma or by synthesis within the tissue. Recent experiments with germ-free rats have demonstrated unequivocally that rat and mouse tissues can form radioactive inositol from [^{14}C]glucose (N. Freinkel & R. M. C. Dawson, unpublished work). However, when tritium-labelled inositol was injected intravenously into rats, a rapid accumulation of the labelled inositol was observed in certain of the tissues, e.g. liver and kidney. This indicates that transport of inositol from the plasma into the tissues can occur against a large concentration gradient and suggests that part at least of the tissue inositol is of dietary origin. Once inside the tissues the metabolism of the inositol can vary markedly, being, for example, high in the liver and kidney and low in the thyroid and pituitary (R. M. C. Dawson & N. Freinkel, unpublished work).

The marked increase of free inositol in the mammary gland during lactation presumably represents an accumulation from the plasma, so that the gland can secrete the comparatively large amounts of free inositol found in milk (Nixon,

1953). The increase cannot be accounted for merely by the presence of non-secreted milk within the gland. Both Climenko & McChesney (1942) and Bowland, Ensminger & Cunha (1948) have reported a beneficial effect of inositol on the lactation of the albino rat. The significance of the drop in the maternal plasma concentration of inositol immediately following parturition is unknown. It cannot be accounted for by the increase in plasma volume which occurs in the lactating rat as this takes place at a later period *post partum* (Bond, 1948, 1958). Although feeding experiments have not demonstrated the importance of dietary inositol in the reproduction of the rat (Ershoff & McWilliams, 1943), Schweigert (1948) has found that, with hamsters on an inositol-deficient diet, females tended to die during parturition and many of the young were born dead or failed to survive. It is possible therefore that parturition is accompanied by an abnormal requirement for inositol on the part of the mother. It is well known that the inositol concentration in foetal blood and fluids is very high compared with those of the mother (Campling & Nixon, 1954), but no evidence has been obtained that this represents active placental transport.

The physiological significance of the high concentrations of inositol in tissues is at the moment obscure. Although it is by no means easy to demonstrate its effect on the growth rate of animals (see, for example, Fenton, Cowgill, Stone & Justice, 1950), there is little doubt that it is a vital factor for the growth of isolated tissue cells in culture (Eagle, Oyama, Levy & Freeman, 1957). At the same time it can be fairly rapidly metabolized by certain tissues, presumably through the glucuronic acid pathway (Charalampous, 1959; Eisenberg, Dayton & Burns, 1959).

The present results suggest that there is a tendency for high inositol levels to occur in organs which are secretory, and in this connexion Hokin & Hokin (1958), Hokin, Hokin, Saffran, Schally & Zimmermann (1958) and Freinkel (1957) have found that the synthesis of inositol phospholipids increases markedly during the secretory activity of endocrine and exocrine glands.

SUMMARY

1. The free inositol contents of the tissues of the rat, guinea pig and rabbit have been measured by a microbiological-assay method.
2. The highest concentrations were found in the thyroid and pituitary glands of all species. In homogenates of these glands in the rat, the inositol was exclusively located in the cytoplasmic fraction obtained by centrifuging.
3. In the organs of all species the concentration

of free inositol was much higher than that found in the plasma.

4. The free inositol content of the rat mammary gland increases markedly during lactation. The concentration in the blood plasma and kidney falls slightly after parturition, but that in the liver does not change.

This investigation was supported in part by Research Grant A-1571 and Training Grant 2A-5060, National Institute of Arthritis and Metabolic Diseases, United States Public Health Service, Bethesda, Md., U.S.A., and by the Capps Fund of Harvard University, and the Wellcome Trust, London. The valuable help of Mr R. B. White in the inositol assays is acknowledged.

REFERENCES

- Bond, C. F. (1948). *Endocrinology*, **43**, 180.
 Bond, C. F. (1958). *Endocrinology*, **63**, 285.
 Bowland, J. P., Ensminger, M. E. & Cunha, T. J. (1948). *Arch. Biochem.* **16**, 257.
 Campling, J. D. & Nixon, D. A. (1954). *J. Physiol.* **126**, 71.
 Charalampous, F. C. (1959). *J. biol. Chem.* **234**, 220.
 Climenko, D. R. & McChesney, E. W. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 157.
 Eagle, H., Oyama, V. I., Levy, M. & Freeman, A. E. (1957). *J. biol. Chem.* **226**, 191.
 Eisenberg, F., Dayton, P. G. & Burns, J. J. (1959). *J. biol. Chem.* **234**, 250.
 Ershoff, B. H. & McWilliams, H. B. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 227.
 Fenton, P. F., Cowgill, G. R., Stone, M. A. & Justice, D. H. (1950). *J. Nutr.* **42**, 257.
 Freinkel, N. (1957). *Endocrinology*, **61**, 448.
 Freinkel, N., Dawson, R. M. C., Ingbar, S. H. & White, R. W. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 549.
 Hamilton, J. W. & Hogan, A. G. (1944). *J. Nutr.* **27**, 213.
 Hartree, E. F. (1957). *Biochem. J.* **66**, 131.
 Hecht, L. I., Stephenson, M. L. & Zamecnik, P. C. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 505.
 Hokin, L. E. & Hokin, M. R. (1958). *J. biol. Chem.* **233**, 805.
 Hokin, M. R., Hokin, L. E., Saffran, M., Schally, A. V. & Zimmermann, B. U. (1958). *J. biol. Chem.* **233**, 811.
 Kojima, Y. & Kusakabe, H. (1954). *J. sci. Res. Inst., Tokyo*, **48**, 121, 284.
 Lovern, J. A. & Olley, J. (1953). *Biochem. J.* **55**, 686.
 Mann, T. (1954). *Proc. Roy. Soc. B*, **142**, 21.
 Meyer, A. E. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 111.
 Nixon, D. A. (1953). *J. Physiol.* **120**, 27P.
 Platt, B. S. & Glock, G. E. (1943). *Biochem. J.* **37**, 709.
 Rickenberg, H. V., Cohen, G. N., Buttin, G. & Monod, J. (1956). *Ann. Inst. Pasteur*, **91**, 829.
 Rodnight, R. (1956). *Biochem. J.* **63**, 223.
 Schweigert, B. S. (1948). *Vitam. & Horm.* **6**, 55.
 Sloane-Stanley, G. H. (1953). *Biochem. J.* **53**, 613.
 Sonne, S. & Sobotka, H. (1947). *Arch. Biochem.* **14**, 93.
 Taylor, A., Pollack, M. A. & Williams, R. J. (1942). *Univ. Tex. Publ.* no. 4237, p. 41.
 Taylor, W. E. & McKibbin, J. M. (1953). *J. biol. Chem.* **201**, 609.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Woolley, D. W. (1940). *Science*, **92**, 384.
 Woolley, D. W. (1942). *J. exp. Med.* **75**, 277.