Vitamin E and stress

5*. The effect of high and low oxygen tension on the metabolism of [^{14}C]D-α-tocopherol in the vitamin E-deficient rat

BY M. A. CAWTHORNE, A. T. DIPLOCK, I. R. MUTHY, J. BUNYAN, ELSPETH A. MURRELL AND J. GREEN

Walton Oaks Experimental Station, Vitamins Ltd, Tadworth, Surrey

(*Received* 20 *December* 1966—*Accepted* 14 *February* 1967)

1. Vitamin E-deficient rats were found to be more susceptible than vitamin E-supplemented controls to the toxic effects of hyperbaric oxygen (60 lb/in.^{2} for 20 min). This agrees with the findings of other workers.

2. Hyperbaric O_2 treatment did not increase the metabolic destruction of a small amount (46·65 μg) of [^{14}C-5-*Me*]D-α-tocopherol given to adult vitamin E-deficient rats 24 h previously. The O_2 treatment also did not affect the soluble sulphydryl compounds and ascorbic acid of rat liver, nor the percentage haemolysis in vivo of rat blood.

3. Hyperbaric O_2 treatment did not increase the true lipid peroxide content of rat brain, compared to control rats treated with hyperbaric air, which has no toxic effects. Increases in 'lipid peroxidation' reported by previous workers are considered to have been due to the use of inadequate controls (untreated rats) and of in vitro techniques that are open to criticism.

4. The toxic effects of hyperbaric O_2 in the vitamin E-deficient rat cannot be attributed to peroxidation in vivo.

5. Vitamin E was not found to protect rats against the effects of reduced O_2 tension (anoxic anoxia). This finding contrasts with some reports by earlier workers. Reduced O_2 tension had no effect on the metabolism of radioactive tocopherol, on blood haemolysis in vivo, or on the soluble sulphydryl compounds and ascorbic acid of liver.

Hyper- and hypo-baric oxygen are among the many stresses that have been reported to affect the vitamin E-deficient animal more than controls. The original discovery of the phenomenon seems to have been made by Puig Muset & Valdecasas (1946) but evidently did not become widely known. It was rediscovered by Taylor (1953), who later made several studies of the subject (Taylor, 1956, 1957, 1958*a*, *b*). He found that toxic effects were more common in vitamin E-deficient rats subjected to O_2 at 55–115 lb/in.^{2} for up to 60 min than in vitamin E-supplemented controls. These effects included convulsions, lung haemorrhage, blood haemolysis in vitro and death. Dietary methylene blue or glutathione decreased the effect on the central nervous system, but they were less active than α-tocopherol. Methylene blue did not affect the degree of lung damage. Gerschman and her colleagues observed similar phenomena and obtained protection against high pressure O_2 poisoning not only with tocopherol and glutathione, but also with cysteine and β-mercapto-ethylamine (Gerschman, Gilbert, Nye, Dwyer & Fenn, 1954; Gerschman, Gilbert, Nye & Fenn, 1954, 1955). They drew an analogy between O_2 poisoning and radiation poisoning, postulating that exposure to hyperbaric O_2 produced free radicals such as the hydroxyl radical, and, subsequently, hydrogen peroxide. The effect of tocopherol and other antioxidants on

* Paper no. 4: *Br. J. Nutr.* (1967), **21**, 497.

hyperbaric O_2 toxicity was apparently rediscovered by Jamieson & van den Brenk (1964) and Kann, Mengel, Smith & Horton (1964), without reference to the earlier work of either Taylor or Gerschman.

An interesting development was the finding by Hove, Hickman & Harris (1945) that vitamin E-deficient rats were more susceptible to hypoxia (anoxic anoxia) than supplemented controls. Telford, Wiswell & Smith (1954) confirmed the effect with rabbits.

In this paper we present results of a study of the effects of both high and low O_2 tension on the survival of vitamin E-deficient rats, on the metabolism of [^{14}C]D-α-tocopherol, and on the concentration of some endogenous hydryl compounds.

EXPERIMENTAL AND RESULTS

General methods

Animals and diets. Rats of the Norwegian hooded strain were used. They were weaned at 21 days and then given a vitamin E-deficient diet until they were 3–5 months of age. The deficient diet A10Y3 is that of Bunyan, McHale & Green (1963). Diet G15F had the composition: casein ('low vitamin content'; Genatosan Ltd) 15, salt mixture 5·13, vitamin mixture (Bunyan, Green, Diplock & Robinson, 1967) 0·4, sugar 54, glucose 20·4 and lard 5. The salt mixture supplied (g/kg): $NaH_2PO_4.2H_2O$ 24, $CaCO_3$ 18·2, KCl 3·5, Na_2CO_3 1·2, $MgSO_4.7H_2O$ 4, ferric citrate 0·15, $MnSO_4.4H_2O$ 0·2, $ZnSO_4.7H_2O$ 0·06, KI 0·0003, NaF 0·00025, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0·002, $CoSO_4.7H_2O$ 0·01, $Al_2SO_4.K_2SO_4.24H_2O$ 0·0007, $CuSO_4.5H_2O$ 0·02 and Na_2SeO_3 0·00007.

High O_2 tension. The general conditions described by Taylor (1956, 1958*a*, *b*) were used. Rats, usually in pairs, were exposed to pure O_2. The pressure was raised to 60 lb/in.^{2} at a rate of 10 lb/in.^{2} per min, and maintained for 20 min (when convulsions usually occurred). The animals were decompressed to atmospheric pressure at a rate of 10 lb/in.^{2} per min. They were then either kept in cages and their time of survival recorded or (in the experiments with radioactive tocopherol) killed at once.

Anoxic anoxia. The general conditions of Hove *et al.* (1945) were used. Rats, usually in pairs, were placed in a large vacuum desiccator and the pressure was reduced at a rate of 3·3 cm Hg/min.

Analytical methods. [^{14}C-5-*Me*]D-α-tocopherol was given orally to rats 24 h before they were subjected to the variation in O_2 tension. [^{14}C]α-Tocopherol in tissues was measured, after chromatographic separation, by scintillation counting according to the methods previously described (Green, Diplock, Bunyan, McHale & Muthy, 1967). Soluble sulphydryl (SH) groups and ascorbic acid in liver were measured as described previously (Green *et al.* 1967). Percentage haemolysis in blood collected into citrate–saline (1·0%; 0·9%) immediately after killing the rats was measured in the supernatant liquid, after centrifuging, by its absorbancy at 560 nm and comparing the latter with the absorbancy of the supernatant liquid completely haemolysed by adding water. Lipid peroxides in adipose tissue and brain were measured by the micro-iodimetric method of Bunyan, Murrell, Green & Diplock (1967). Malondialdehyde

determinations in brain tissue were by the new method of Placer, Cushman & Johnson (1966). The tissue (0·5 g) was homogenized for only 30 secs at 1000 rev/min in a nylon–glass homogenizer with 20 ml 0·9% NaCl solution, buffered at pH 7·4. Malondialdehyde bis-bisulphite sodium salt, with a molecular extinction coefficient of $1{\cdot}49 \times 10^5$ (Placer *et al.* (1966) give $1{\cdot}52 \times 10^5$), was used as standard.

Experiments with hyperbaric O_2

Expt 1. The effect of vitamin E in protecting rats against toxicity due to high O_2 tension was studied. Vitamin E-deficient 5-month-old female rats were compared with control rats that had received the deficient diet A10Y3 supplemented with 120 ppm D-α-tocopheryl acetate from weaning. Two rats (one from each group) were exposed together to hyperbaric O_2 for 20 min; after decompression their survival time was measured. Six rats from each group were examined and the results are shown in Table 1. All the deficient rats died within the next 3 days. *Post mortem* they showed signs of haemorrhagic renal congestion. All the rats in the supplemented group survived and appeared normal after 3 days. Similar tests were made on groups of deficient and supplemented rats, using air at 60 lb/in.^{2} in place of oxygen. The rate of decompression was reduced at 5 lb/in.^{2} per min to minimize nitrogen 'bends'. No toxicity was observed in either group.

Table 1. *Expt* 1*: the effect of vitamin E on the survival time of female rats subjected to hyperbaric air or oxygen*

(5-month-old vitamin E-deficient rats were compared with controls given D-α-tocopheryl acetate (120 ppm) in the diet from weaning. A rat from each group was subjected to 60 lb/in.^{2} air or oxygen for 20 min and then decompressed at 10 lb/in.^{2} min)

Group	Treatment	Rat wt* (g)	No. in group	Time of death† (h)	No. of survivors after 3 days
Vitamin E-deficient	Oxygen	166 ± 7	6	3½, 5½, 12, 16, 60, 60	0
Vitamin E-supplemented	Oxygen	169 ± 19	6	—	6
Vitamin E-deficient	Air	154 ± 15	6	—	6
Vitamin E-supplemented	Air	163 ± 25	6	—	6

* Mean with standard deviation.
† Measured from time at which 60 lb/in.^{2} was reached.

Expt 2. In this experiment some biochemical effects of hyperbaric O_2 were studied. Sixteen male rats that had received diet A10Y3 until they were 3 months of age were each given a single oral dose of 46·65 μg (5696 disintegrations/sec (dps)) [^{14}C-5-*Me*]-D-α-tocopherol. They were divided into two groups of eight. Rats of group 1 were subjected to hyperbaric O_2 24 h later. After decompression the rats were killed by severing their heads and blood was at once collected. Percentage haemolysis and [^{14}C]α-tocopherol were determined in separate portions. The livers were removed and used for the determination of soluble SH compounds and ascorbic acid. The carcasses, after removal of the gastro-intestinal tract, were analysed for [^{14}C]α-tocopherol. At the same time, rats of group 2, not treated with hyperbaric O_2 were killed

https://doi.org/10.1079/BJN19670069 Published online by Cambridge University Press

Table 2. *Expt 2: the effect of hyperbaric oxygen on the metabolism of* [^{14}C]*α-tocopherol in 3-month-old vitamin E-deficient male rats and on soluble sulphydryl compounds and ascorbic acid in their livers and percentage haemolysis of their blood*

(Sixteen rats were each given orally 46·65 μg (5696 dps) [^{14}C-5-*Me*]D-α-tocopherol and divided into two groups. After 24 h, rats from group 1 were subjected in pairs to oxygen at 60 lb/in.^{2} for 20 min and then killed. Untreated control rats from group 2 were killed at the same time. Results are given as means with standard deviations. Each analysis (four per group) was carried out on the combined tissues from each pair. The haemolysis measurements were carried out on the blood of each rat separately)

		Blood		Carcass†	Liver	
Group	Rat wt* (g)	Haemolysis (%)	[^{14}C]α-tocopherol (dps/100 ml)	[^{14}C]α-tocopherol (total dps)	Soluble SH (μ-equiv./g)	Ascorbic acid (μ-equiv./g)
1 (high O_2 tension)	136·6 ± 44·3	4·14 ± 2·93	106·4 ± 33·5	2161 ± 400	3·18 ± 1·86	3·22 ± 1·02
2 (untreated controls)	138·0 ± 47·3	2·41 ± 1·97	60·3 ± 14·4	2458 ± 743	3·80 ± 1·51	2·65 ± 0·58

* Mean with standard deviation. † With intestinal tract removed.

and analysed similarly. Haemolysis was recorded on the blood of each rat separately, but for all the other analyses tissues were combined in pairs. The results are given in Table 2. Treatment with hyperbaric O_2 had no significant effect on any of the quantities measured.

Expt 3. Kann *et al.* (1964) regarded the toxic effects of hyperbaric O_2 as being due to 'lipid peroxidation'. More recently, a report from the same laboratory has suggested that subjection of vitamin E-deficient mice to high O_2 tension increases the 'lipid peroxide' content of the forebrain (Zirkle, Mengel, Horton & Duffy, 1965). They claim that 'peroxide' formation in the brain is associated with the incidence of convulsions and conclude that 'the ultimate results of hyperoxic pressure on cells and tissues in the central nervous system reflect the toxic effects of lipid peroxides at multiple metabolic sites'. Because of the contrary indications found in Expt 2 above, we have investigated the situation in brain in more detail.

Female rats were given the vitamin E-deficient diet G 15 F until they were 4 months of age. They were then subjected in pairs to hyperbaric O_2 under the conditions of Expts 1 and 2. Following the procedures of Zirkle *et al.* (1965) the rats were killed immediately after decompression and their brains removed (in one experiment the forebrains were isolated for study). However, unlike Zirkle *et al.* (1965), who killed by decapitation and removed the tissue in air, we killed the rats by asphyxiation with nitrogen and all subsequent treatment of the tissues was carried out in N_2; also, we measured true lipid peroxides by the micro-iodimetric method of Bunyan, Murrell *et al.* (1967). At the same time, following Zirkle *et al.* (1965), two untreated control rats were killed and their tissues were examined. The results are given in Table 3. The true lipid peroxide content of all brains, whether treated with hyperbaric O_2 or not, was very low (cf. the normal peroxide contents of other tissues, including those from vitamin E-supplemented animals, as given by Bunyan, Murrell *et al.* (1967)). The mean peroxide value of the O_2-treated brains was slightly higher than that of the controls; statistical examination by the method of paired comparisons showed that the difference between the means was significantly different from zero ($P < 0{\cdot}05$, one-sided t test). In one test, samples of adipose tissue were also examined for peroxides. As shown in Table 3, the values were similar to those for brain, although again the value for the treated tissue was higher than that of the untreated control. However, Horgan & Philpot (1961) have shown that the concentration of a peroxide-like substance is increased in rats that have been alarmed. It occurred to us that such a factor might operate in these tests, since the control rats had not been subjected to the stresses of handling and compression. Accordingly, two more tests were made, and in these the control rats were put through a similar procedure to the test rats, but were exposed to hyperbaric air rather than O_2, at 60 lb/in.^{2} As Table 3 shows, the brains of these air-treated rats contained the same amount of lipid peroxide as did the brains of the O_2-treated rats.

Zirkle *et al.* (1965) measured, not true peroxides, but the malondialdehyde formed by reaction of tissue extracts with 2-thiobarbituric acid (TBA). We also measured malondialdehyde formation in some of the brains in order to compare our results (Table 3) directly with those of Zirkle *et al.* (1965). Malondialdehyde values at zero

Table 3. *Expt 3: lipid peroxides and 'lipid peroxidation' in brain and adipose tissue of 4-month-old vitamin E-deficient female rats treated with hyperbaric oxygen compared with untreated controls and controls treated with hyperbaric air*

(The rats were treated in pairs with O_2 or air at 60 lb/in.^{2} for 20 min. They were then killed in N_2 and their brains were analysed at once. Untreated controls were taken from their cages and killed at the same time as the corresponding O_2-treated pair. The results of each test (on the combined tissues of two rats) are given individually)

		Lipid peroxide (μ-equiv./g)			Malondialdehyde (μg/g)			
					O_2-treated rats		Untreated rats	
Test	Tissue	O_2-treated rats	Air-treated rats	Untreated rats	Zero time	After 30 min at 37°*	Zero time	After 30 min at 37°*
1	Brain	1·04	—	0·49	—	—	—	—
2	Brain	1·83	—	1·02	10·6	38·7	9·2	33·0
3	Forebrain†	0·69	—	0·35	6·9	26·5	7·8 (12·4‡)	24·6
	Adipose tissue	0·45	—	0·24	—	—	—	—
4	Brain	0·45	0·55	—	—	—	—	—
5	Brain	0·85	0·86	—	—	—	—	—

* Incubated in a volume of 1·5 ml in a 10 ml test-tube and shaken at 100 agitations/min.
† Forebrain blended in solvent and then N_2 passed through for 3 min to remove any adhering or dissolved O_2.
‡ Value after allowing the homogenate to stand at 0° for 12 min after preparation.

Table 4. *Expt 5: the effect of anoxic anoxia on the metabolism of [^{14}C]α-tocopherol in 3½-month-old vitamin E-deficient female rats and on soluble sulphydryl compounds and ascorbic acid in their livers and on percentage haemolysis of their blood*

(Sixteen rats were each given orally 52·5 μg (6290 dps) [^{14}C-5-*Me*]D-α-tocopherol and divided into two groups. After 24 h, rats from group 1 were subjected in pairs to low oxygen tension at 30 cm Hg for 12 min and then killed. Control rats from group 2 were killed, without exposure, at the same time. Results are given as means with standard deviations. Each analysis (four per group) was carried out on the combined tissues from each pair. The haemolysis measurements were carried out on the blood of each rat separately)

		Blood		Carcass		Liver	
Group	Rat wt (g)	Haemolysis (%)	[^{14}C]tocopherol (dps/100 ml)	[^{14}C]tocopherol (total dps)	[^{14}C]metabolites (total dps)	Soluble SH (μ-equiv./g)	Ascorbic acid (μ-equiv./g)
1 (low O_2 tension)	93·5 ± 34·0	1·45 ± 0·60	688 ± 452	2266 ± 652	663 ± 283	2·56 ± 0·96	1·99 ± 0·35
2 (controls)	92·5 ± 33·0	2·04 ± 1·66	318 ± 151	2342 ± 652	479 ± 226	2·12 ± 0·66	1·75 ± 0·11

time and after incubation in vitro rose by no more than 17% in the O_2-treated tissues, compared with untreated controls.

Experiments with hypobaric O_2

Expt 4. The ability of vitamin E to protect rats against the effects of anoxic anoxia was examined. Fifteen female rats were given the vitamin E-deficient diet G15F for 4 months. Seven of these rats were then transferred to the same diet supplemented with D-α-tocopheryl acetate, 100 ppm. The remaining eight rats continued to receive diet G15F. After 4 weeks on these diets, the rats (mean weight 155 g) were subjected to low O_2 tension (for conditions, see p. 672). Two rats (one from each group) were placed together in the apparatus for each exposure, and their time of survival was measured, the index of death being cessation of breathing. The maximum exposure time was 75 min, and one rat from each group survived for this time. The survival time (mean with standard deviation) for rats in the vitamin E-deficient group was 23 ± 15 min, whilst for rats in the vitamin E-supplemented group it was 7 ± 8 min. Statistical analysis showed that there was no significant difference between these means.

Expt 5. The effect of anoxic anoxia on erythrocyte haemolysis, soluble SH compounds and ascorbic acid in liver and on the metabolism of α-tocopherol was examined. Sixteen $3\frac{1}{2}$-month-old vitamin E-deficient female rats were each given orally 52·5 μg (6290 dps) [^{14}C-5-*Me*]D-α-tocopherol. After 24 h, eight rats were subjected in pairs to anoxic conditions. They were maintained at 30 cm Hg for 12 min, after which time pressure was restored to atmospheric at a rate of 10 cm Hg/min. The rats were then killed by breaking their necks and blood was collected as described in Expt 2. At the same time, four pairs of control rats were killed without being exposed to anoxia, and their blood was taken. Samples of liver were combined from each pair of rats and used to determine soluble SH compounds and ascorbic acid. The remainder of the carcasses and samples of blood were analysed for radioactive α-tocopherol. The results are shown in Table 4. There were no significant differences between groups in any of the quantities measured.

DISCUSSION

In agreement with earlier workers, exposure of vitamin E-deficient rats to O_2 at 60 lb/in.^{2} resulted in convulsions and death; vitamin E was completely protective. However, in contrast to Taylor (1956, 1957, 1958*a*, *b*), we did not find greatly increased erythrocyte haemolysis in vivo in the vitamin E-deficient animals. If the protocols of Taylor's various experiments are examined, it seems that his haemolysis test 'in vivo' refers to a measurement of dialuric acid-induced haemolysis in vitro (Rose & György, 1950) after the exposure of a sample of the rats' blood to O_2 during the experimental compression of the rats.

Although in our experiments the protective effect of tocopherol is clear, we were unable to find any evidence that the toxic effect of O_2 involves direct 'oxidation' reactions. Rats given a small amount of radioactive α-tocopherol 24 h before exposure to hyperbaric O_2 contained the same quantities of radioactive tocopherol in their

blood and carcasses after death as did controls not exposed. In a parallel fashion, although Taylor (1957, 1958*a*) found that glutathione protects rats against hyperoxia, we found (in Expt 2) that soluble SH compounds in liver were unaffected by O_2 tension. No effect on ascorbic acid was found.

These results agree with our previous findings that stress conditions, even those usually considered to be 'oxidative' in nature, do not appear to lead to an increase in oxidation reactions in vivo. They reinforce our opinion that current concepts of uncontrolled and extensive peroxidation of lipids in vitamin E-deficient animals are largely in error. The recent statement by Zirkle *et al.* (1965) that exposure to hyperbaric O_2 gives rise to 'lipid' peroxidation in the brains of mice and that this is causally concerned with the incidence of convulsions was therefore of special interest. Several criticisms can be made of their report. The TBA method measures not true peroxides but malondialdehyde, the formation of which in vitro is probably unrelated to the situation in vivo (Bunyan, Murrell *et al.* 1967). Moreover, the use of a 10 min homogenization period by Zirkle *et al.* (1965) would give values for malondialdehyde greater than zero time values to a degree that would obliterate any small effect at zero time due to the O_2 treatment. As shown in our Table 3, even allowing the homogenate to stand exposed to air for 12 min leads to a 60% increase in TBA value, which is much more than the 15% difference between O_2-treated and untreated brain at zero time. When we determined the true peroxide content of rat brain we found only a slight effect in the O_2-treated animals, which was shown to be due, not to the O_2, but to the handling of the animals and the drastic experimental procedure (cf. Horgan & Philpot (1961) who found an increase in a peroxide-like substance in rats due to an alarm response).

The difference in malondialdehyde production found by Zirkle *et al.* (1965) between the brains of vitamin E-deficient and supplemented mice is irrelevant to the problem of peroxidation in vivo: it merely demonstrates that α-tocopherol inhibits autoxidation in vitro, a process that would readily occur during their 10 min homogenization time. The irrelevance of such in vitro procedures as a means of measuring peroxidation in vivo has been discussed by Bunyan, Murrell *et al.* (1967), who have shown that vitamin E, in fact, does not affect the true peroxide content of tissues other than adipose tissue. Although the role of vitamin E in protecting against physiological stress is still far from being understood, it can now be regarded as virtually certain that the mechanism does not involve either the inhibition of peroxidation reactions or the destruction of tocopherol. In a recent communication, Kokatnur, Bergan & Draper (1966) have given yet a further example of a stress (intravenous infusion of methyl linoleate hydroperoxide) that can be largely overcome by vitamin E. Their suggestion, without the support of analytical evidence, that this stress 'leads to a more rapid destruction of vitamin E in the tissues' we would regard as unacceptable.

We were unable to repeat satisfactorily the work of Hove *et al.* (1945), who found that vitamin E protected rats against the effects of low O_2 tension. We found that anoxic anoxia did not affect the metabolism of [^{14}C]α-tocopherol in rats, or the amount of soluble SH compounds and ascorbic acid in their livers.

REFERENCES

Bunyan, J., Green, J., Diplock, A. T. & Robinson, D. (1967). *Br. J. Nutr.* **21**, 137.
Bunyan, J., McHale, D. & Green, J. (1963). *Br. J. Nutr.* **17**, 391.
Bunyan, J., Murrell, E. A., Green, J. & Diplock, A. T. (1967). *Br. J. Nutr.* **21**, 475.
Gerschman, R. Gilbert, D. L., Nye, S. W., Dwyer, P. & Fenn, W. O. (1954). *Science, N.Y.* **119**, 623.
Gerschman, R., Gilbert, D. L., Nye, S. W. & Fenn, W. O. (1954). *Proc. Soc. exp. Biol. Med.* **86**, 27.
Gerschman, R., Gilbert, D. L., Nye, S. W. & Fenn, W. O. (1955). *Fedn Proc. Fedn Am. Socs exp. Biol.* **14**, 56.
Green, J., Diplock, A. T., Bunyan, J., McHale, D. & Muthy, I. R. (1967). *Br. J. Nutr.* **21**, 69.
Horgan, V. J. & Philpot, J. St L. (1961). *Nature, Lond.* **192**, 662.
Hove, E. L., Hickman, K. & Harris, P. L. (1945). *Archs Biochem.* **8**, 395.
Jamieson, D. & van den Brenk, H. A. S. (1964). *Biochem. Pharmacol.* **13**, 159.
Kann, H. E. Jr, Mengel, C. E., Smith, W. & Horton, B. (1964). *Aerospace Med.* **35**, 840.
Kokatnur, M. G., Bergan, J. C. & Draper, H. H. (1966). *Proc. Soc. exp. Biol. Med.* **123**, 254.
Placer, Z. A., Cushman, L. L. & Johnson, B. C. (1966). *Analyt. Biochem.* **16**, 359.
Puig Muset, P. & Valdecasas, F. G. (1946). *Trab. Inst. nac. Cienc. méd., Madr.* **6**, 389.
Rose, C. S. & György, P. (1950). *Proc. Soc. exp. Biol. Med.* **74**, 411.
Taylor, D. W. (1953). *J. Physiol., Lond.* **121**, 47*P*.
Taylor, D. W. (1956). *J. Physiol., Lond.* **131**, 200.
Taylor, D. W. (1957). *J. Physiol., Lond.* **135**, 60*P*.
Taylor, D. W. (1958*a*). *J. Physiol., Lond.* **140**, 23.
Taylor, D. W. (1958*b*). *J. Physiol., Lond.* **140**, 37.
Telford, I. R., Wiswell, O. B. & Smith, E. L. (1954). *Proc. Soc. exp. Biol. Med.* **87**, 162.
Zirkle, L. G. Jr, Mengel, C. E., Horton, B. D. & Duffy, E. J. (1965). *Aerospace Med.* **36**, 1027.

Printed in Great Britain