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Adaptation of Mice to Carbon Monoxide and the Effect of Splenectomy

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Summary. The adaptation of normal and splenectomized mice to increasing concentrations of carbon monoxide (CO) in air, and their subsequent long-term survival in it were studied. From the 10th day onwards the concentration of CO was maintained at 0.24%, which produced a carboxyhaemoglobin level of about 74% in the blood, and which was normally lethal within 24 h. However, the non-splenectomized mice survived in this environment for an average of 47 days, but splenectomized mice survived for 101 days.

During adaptation to CO there were marked increases in the haematocrit level and the concentration of haemoglobin, a massive release of reticulocytes into the circulation, and increases in blood volume, spleen and heart weight. However, changes in the above haematological parameters were significantly less in splenectomized than in normal animals. These differences were attributed to the loss of the erythropoietic reserves of the spleen.

In splenectomized mice which survived for over 90 days in CO there was histological evidence for the development of extramedullary erythropoiesis, as well as increased erythropoietic activity in the marrow of the tail vertebrae, which occurred earlier. Deaths were most frequently associated with massive internal haemorrhages in both groups of animals. This is discussed in relation to histological and haematological findings. Also in both groups, there was evidence that both hypertrophy and hyperplasia of heart muscle fibres occurred during adaptation to breathing CO.

Key words: Carbon monoxide — Splenectomy — Erythropoiesis — Heart muscle — Mice.

Introduction

The increasing role of carbon monoxide (CO) in industry has prompted considerable research into its biological and pathological effects (Theodore, O'Donnell and Back, 1971; Abidin et al., 1973; Utidjian, 1973; Thomsen and Kjeldsen, 1973), including those on the cardiovascular system, and especially the coronary circulation (Ayers, Gianelli and Mueller, 1970; Astrup, 1972a, b; Adams, Erickson and Stone, 1973; Anderson et al., 1973; DeBias et al., 1973; Stewart et al., 1973).

An important finding has been that the body is able to adapt in various ways to the partial saturation with CO of circulating haemoglobin. One major type of adaptation is haemodynamic; cardiac output is increased. We found that in exposed mice the volume of the blood is very much increased. Another is haemopoietic; in effect this increases the amount of haemoglobin in the blood. We were particularly interested in the haemopoietic adaptation of mice to CO after splenectomy, since we did not find references on this subject but it is known that a significant proportion of erythrocytes normally originate in the spleen

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(Aggio and García, 1969; Aggio, 1973). Our results show different haematological parameters in normal and splenectomized mice followed up for long periods of exposure. We also followed changes in heart weight, and examined the cardiac muscle fibres for evidence of hypertrophy of hyperplasia, in mice subjected to prolonged exposure to near-lethal concentrations or CO, since this is a point of controversy.

The possible causes of the considerable extension of life of splenectomized, as opposed to non-splenectomized mice, are discussed.

Materials and Methods

The *experimental animals* were random-bred female albino mice, weighing 15–26 g, from a closed colony in the animal house of King's College Hospital Medical School. Approximately half the mice were splenectomized under ether anaesthesia at 6 weeks of age; controls were unoperated on. After 14 days mice from each group were placed in 27.5 litre glass chromatography tanks $26 \times 39 \times 27$ cm, each of which contained 10–16 animals in two standard metal cages, where they were exposed to CO.

Carbon monoxide, supplied by the British Oxygen Company, Special Gases Division, London S.W. 19, was passed through a floating bobbin 'Rotameter' flowmeter at an appropriate rate and mixed with air from a small pump. The mixture was then passed through the tank at a flow-rate of 3–6 l/min, depending upon the number of mice in it. The concentration of CO was set initially at 0.05% and was increased stepwise over a period of 10 days to 0.24% (Table 1).

Table 1. Stepwise increase in carbon monoxide concentration

Day	0	1	2	3	4	5	6	7	8	9	10
Concentration of CO (% v/v)	0.05	0.06	0.07	0.08	0.09	0.10	0.12	0.15	0.18	0.21	0.24

This regimen, which was developed empirically by Miss G. M. Baker in this laboratory, is near the maximum tolerable rate of increase. Similar adaptation procedures were followed by Campbell (1933) and by Abidin et al. (1973). Some groups of animals were killed on the 10th or 11th day, while others were kept in 0.24% CO for 42 or 43 days, or until death.

Exposure was either continuous (24 h/day) or the mice were only placed inside the tanks for 4, 8 or 16 h per 24 h period. The animals were removed for 20–30 minutes about three times a week for weighing, and changing the cages. At the times indicated in the graphs blood samples were also taken before replacing the mice. As far as possible, however, these operations were timed to avoid scheduled periods of CO-exposure.

Throughout the experiments the mice were provided with Oxoid 'Diet 41B' animal cubes and tap water *ad libitum*. Experimental and control animals were maintained at room temperature (generally 22–26° C).

Blood samples were obtained from the tail; up to four bleedings of approximately 0.1 ml per animal were performed at intervals of not less than three days.

Total haemoglobin was estimated after diluting samples in Drabkin's solution with a photometric haemoglobin-meter (Vitatron U.K., Ltd.). The percentage of carboxy-haemoglobin was estimated from determinations on fresh and reduced alkaline dilutions at 555 and 480 nm, using a 'Unicam' SP1800 Ultraviolet Spectrophotometer, according to the method of Klendshoj, Feldstein and Sprague (1950). The packed cell volume was determined by microhaematocrit centrifugation. Reticulocytes were counted in smears stained with cresyl violet. In one set of samples the white cells were counted using a Coulter Counter, Model F.

In one experiment, after the completion of exposure to CO, the mice were bled out under ether anaesthesia from the renal artery or the abdominal aorta to estimate the approximate blood volume. Otherwise they were killed by cervical dislocation. The heart and spleen (when still present) were removed and weighed fresh, then these and other organs, including liver, lungs, kidneys, lumbar and tail vertebrae and sternum, were fixed in 10% formol saline for histological examination. In one experiment the freshly removed hearts were pooled and homogenized, and samples were analysed chemically to determine the water, total protein-nitrogen (by the Kjeldahl method) and hydroxyproline (by a spectrophotometric method) content.

The *internal organs*, after fixation, were embedded in paraffin and sections 3 or 5 μm thick were stained with haematoxylin and eosin. The hearts were sectioned transversely across the upper portions of the ventricles. The diameters of the muscle fibres were estimated under × 400 magnification using a ruled eyepiece graticule, by three different methods; (a) by measurement of the cross-sectional diameter of the fibres in one plane, or (b) in two perpendicular planes, or (c) by counting the number of fibres along a known length of the graticule scale. Cross-section measurements were made in the subepicardial and subendocardial layers, since cross-sectioned fibres were present mainly in these areas. The length of the scale in (c) covered nearly the whole thickness of the left ventricular wall or the whole of the right wall. In each heart wall 15 or 32 fibres were measured by method (a), 30 by method (b), and 40–70 by method (c).

The density of nucleated cells in the bone marrow was estimated from the number per unit area under × 950 magnification, using a squared eyepiece graticule. The standard square covered an area of 625 μm^2. Vertebrae were taken from the lumbar region and from three regions of the tail; proximal, middle and distal. The tail vertebrae were cut through the long axis of the marrow cavity and the lumbar vertebrae were cut transversely. Cell counting was done over the whole length of the cavity of the tail vertebrae, but in the sternum and lumbar vertebrae the area was limited to 10–25 graticule squares due to the larger number of cells per unit area.

The *experimental groups* initially contained a minimum of 5 mice, and up to 12 in some cases. Results were calculated and analysed for significance by Student's 't-test' using an Olivetti 'Programma 101' electronic calculator.

Results

The carboxyhaemoglobin content in the blood of animals kept in 0.24% CO showed a value of 74%.

Figure 1 shows reticulocytes in the blood in normal and splenectomized mice kept in air or exposed to CO. The normal value was approximately 3% of red blood cells, but this increased sharply to a peak of 28% after 6 days' exposure to CO, then fell slightly by the 10th day (curve A). In non-splenectomized mice exposed for 21 days before being bled (Curve B) the reticulocyte count was lower than in corresponding groups which were bled during the first 10 days of exposure. It was, however, approximately twice as high as in CO-exposed splenectomized animals (curve D). Similarly, among splenectomized mice bled during the first 10 days' exposure (curve C) the response was about half that of non-splenectomized mice, the difference on any day being highly significant ($P = 0.036$–0.001). This trend persisted throughout the experiment. Splenectomy alone had no significant effect on the reticulocyte counts either after 14 or 55 days (curve F and point G; days 0 and 41 of exposure, respectively). However, bleeding caused the reticulocyte count to rise in air-breathing mice, and splenectomy reduced the magnitude of this effect (curves E and F).

Results from other experiments (Table 2) show that in non-splenectomized mice the increase in the reticulocyte count was approximately proportional to

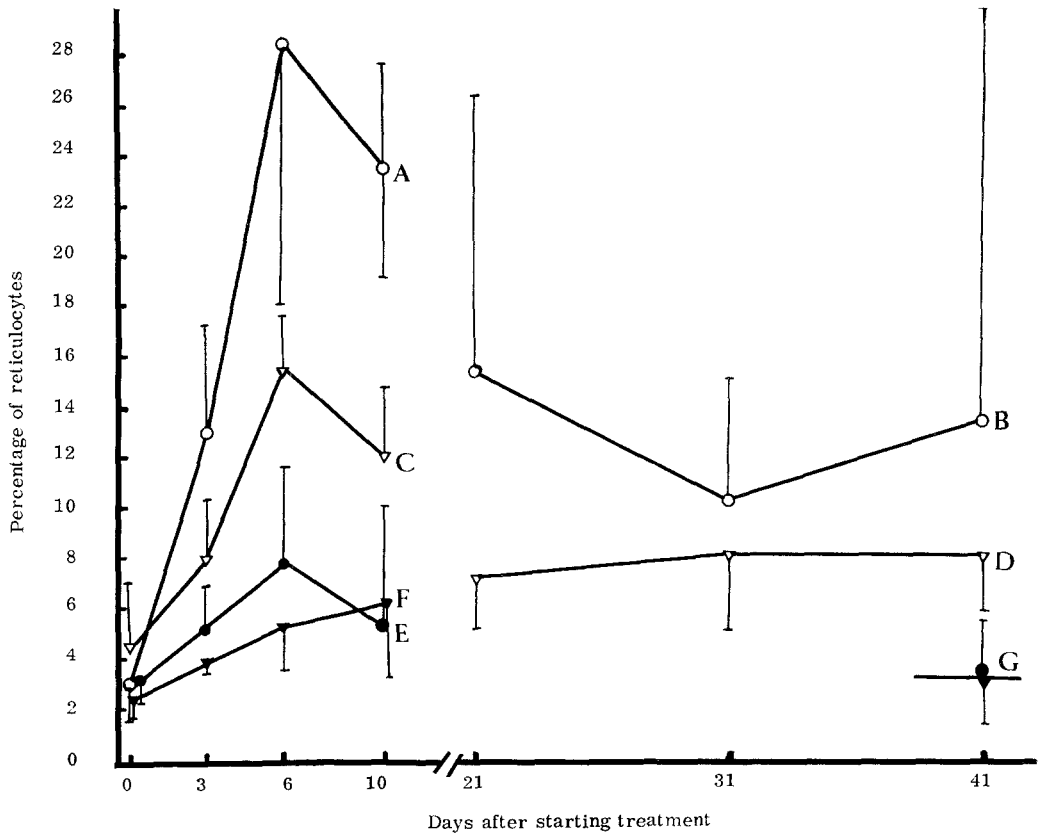

Fig. 1. Effect of splenectomy on blood reticulocytes following CO exposure. Vertical bars represent 95% confidence limits. ○ (A and B): Normal mice in CO. ▽ (C and D): Splenectomized mice in CO. • (E and G): Normal mice in air. ▼ (F and G): Splenectomized mice in air. The curves on the left-hand side of the graph (A, C, E and F) represent groups of animals bled on days 0, 3, 6 and 10 of exposure; those on the right-hand side (B and D) refer to groups bled on days 21, 31 and 41; the two groups represented by points in position G were only bled on day 41

Table 2. Percentage reticulocyte counts on the sixth day of exposure to CO

Length of exposure to CO per 24 h period (hours)	Normal mice	Splenectomized mice
0	A 5.6 ± 0.9[a]	n.d.
	B 8.2 ± 1.2	B 5.4 ± 0.7
4	n.d.	B 10.8 ± 1.7
8	A 13.7 ± 1.1	n.d.
16	A 21.1 ± 2.1	B 12.7 ± 1.6
24	A 17.5 ± 1.5	n.d.

A = first experiment, B = second experiment, n.d. = not done
[a] Mean % reticulocyte count ± standard error

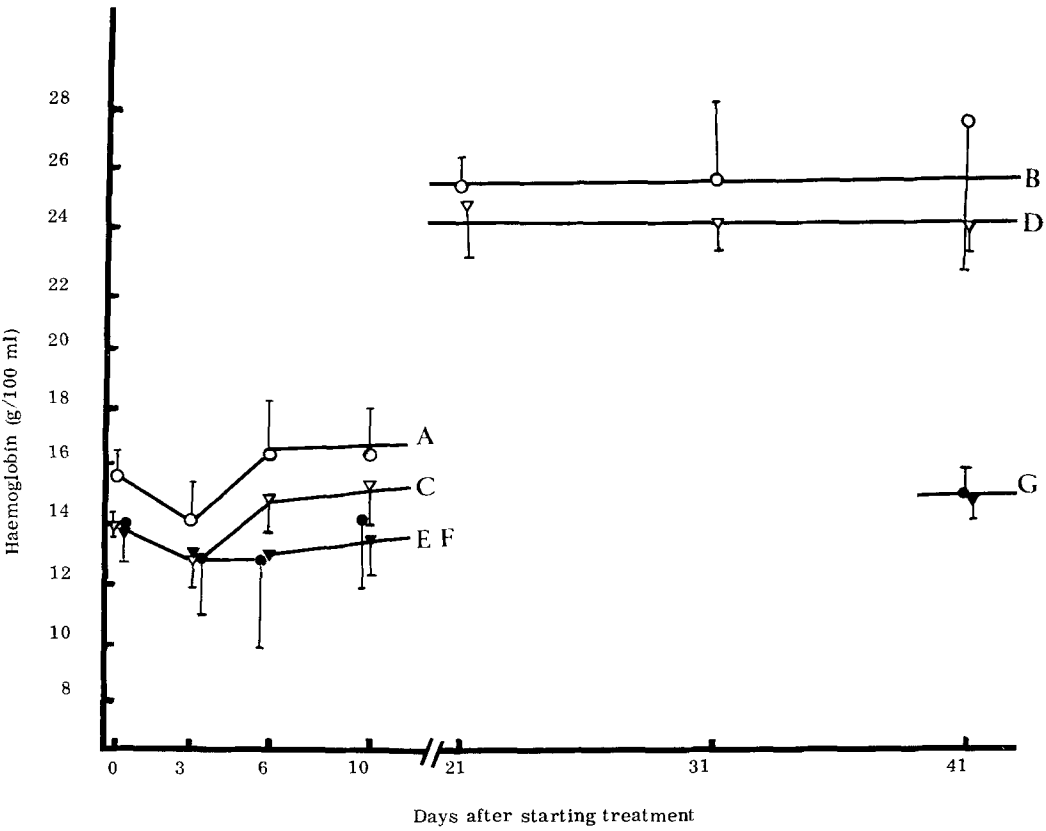

Fig. 2. Effect of splenectomy on blood Hb following CO exposure. Animal groups and symbols as in Figure 1

the length of daily exposure to CO up to a maximum at 16 h per day. The level in the continuously-exposed group was slightly lower, although the difference was not statistically significant. In splenectomized animals there was a similar, but smaller increase in the reticulocyte count with increasing length of daily exposure.

Haemoglobin levels in mice bled on day 0 of exposure to CO showed a slight decrease on the 3rd day and then a gradual increase up to 16.5 g/100 ml on the 10th day (Fig. 2, curve A). This increase was less evident in splenectomized mice (curve C). Splenectomy itself produced a small transient fall in the haemoglobin level after 14 days, but by the 55th day the level was nearly normal (curve F and point G; days 0 and 41 of exposure, respectively).

Curves B and D represent haemoglobin levels in mice which were not bled until the 21st day of exposure. The haemoglobin level of non-splenectomized mice (curve B) approached twice the control value (27.7 g/ml compared to 15.3 g/100 ml) on day 41. While the response in splenectomized mice (curve D) was only slightly less than in non-splenectomized animals on any given day, the difference became highly significant ($P < 0.01$) when all days tested were taken into consideration, and the trend was consistent throughout the experiment.

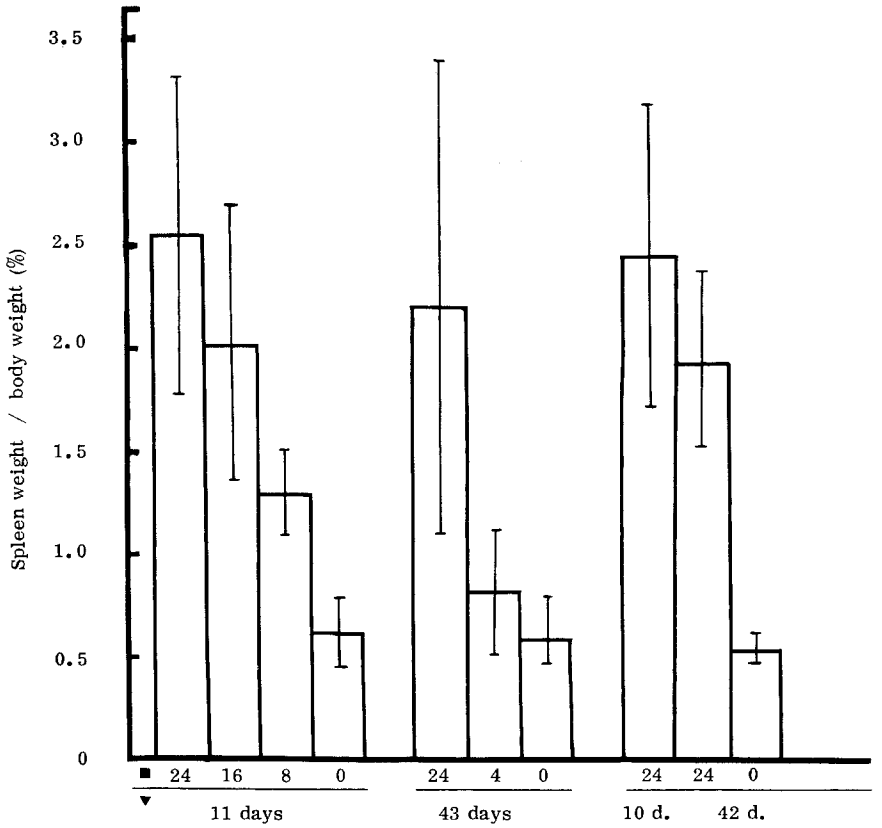

Fig. 3. Spleen weights expressed as percentages of body weight. Figures in the upper line of the abscissa (■) represent period of exposure to CO in hours per day; figures in the lower line (▼) show the number of days of exposure. Each group of columns represents a separate experiment

Exposure to CO for 4 h per day strongly stimulated the production of haemoglobin, as in the case of reticulocytes; longer exposures had little additional effect.

The total packed cell volume (haematocrit) in non-splenectomized non-treated mice was approximately 45%. Continuous exposure of non-splenectomized animals to CO caused a rise to 67% after 10 days of exposure and to 85% after 41 days. The corresponding values in splenectomized animals were only 55% and 79% (a significant difference).

The white cell counts after 41 days of CO exposure were slightly lower, both in normal and splenectomized groups, than in the air-breathing animals. Splenectomy had no apparent effect.

The mean volume of blood obtained from mice treated with CO for 36 days was 3.18 ml compared to only 0.88 ml from controls, which was a highly significant increase ($P \ll 0.001$).

The mice stopped gaining in body weight, or even lost weight, during the first 10 days of exposure. However, growth resumed after 14 days when the level of

CO had become steady at 0.24%. By day 42 the difference between treated groups and controls had largely been made up.

In non-splenectomized mice exposed to CO for 11 days the spleens enlarged nearly 5 times relative to the body weight, i.e. from 0.55% to 2.52% (Fig. 3). This weight increase was proportional to the time of exposure, being greatest in groups exposed for 24 h per day, and least in mice exposed for 8 h per day. Prolonging the experiment to 42 days did not produce any further enlargement of the spleen. An exposure of 4 h per day showed a relative doubling of the spleen weight on day 11 (not shown in graph), but by day 43 it was only 40% larger than in controls (Fig. 3).

Histological sections of spleens from CO-exposed animals revealed blurring of the outlines of lymphatic follicles, with diffuse infiltration of the red pulp by immature round cells containing hyperchromatic nuclei, many of which had features of erythroblasts. These cells were evenly distributed throughout the organ, compressing the sinuses to the exclusion of erythrocytes, and seemed partially to replace the follicles.

Sections through the bone marrow of the tail vertebrae in control mice showed nucleated cells mainly restricted to the ephiphyses (Fig. 4a) and the greater part of the volume was occupied by fatty tissue. Of the nucleated cells, approximately 40% were polymorphonuclear leukocytes (PMN), 40% were stromal cells, including fixed reticulum cells, endothelial cells and fibroblasts, and 20% had round nuclei and included undifferentiated marrow cells and lymphocytes.

Splenectomy and subsequent continuous exposure of mice to CO for 42 days produced a very marked increase in the cellularity of the tail marrow, and at the same time the cells became evenly distributed throughout the vertebral cavity (Fig. 4b). Most of the cells were erythroblasts (inset in Fig. 4).

Figure 5 shows the average number of cells per unit area in the tail vertebrae in various groups of mice. The number of cells in the non-splenectomized groups exposed to CO for 11 or 42 days was similar to controls, i.e. 3. However, splenectomy followed only by blood sampling was sufficient to cause an increase in the cellularity to about 5. This increase was further enhanced to approximately 8 cells per unit area by CO-exposure for 16 h per day for 11 days, and to 14 cells by continuous exposure for 42 days. The latter value was within the range found in the sternum and lumbar vertebrae of controls and CO-exposed mice. The increase in the cellularity of the tail marrow was also accompanied by changes in the relative numbers of different cell-types in CO-exposed groups; PMN fell to 12% and stromal cells to 2%, while cells with round nuclei increased to 86%. The percentages of the various types of cells in the marrow of the sternum were also affected by CO-exposure. In controls PMN accounted for 35% and round-nucleated cells 62%, while in non-splenectomized mice exposed to CO for 10 or 42 days the round-nucleated cells increased to 75%, and in splenectomized animals they reached 80% after 42 days. These increases were mainly at the expense of PMN.

Heart weights of mice are presented in Figure 6. This histogram shows a 100% relative weight increase of the hearts in mice exposed continuously to CO for 11 days. Shorter periods of daily exposure produced proportionally smaller enlargement. Extending the length of exposure to 43 days resulted in a further

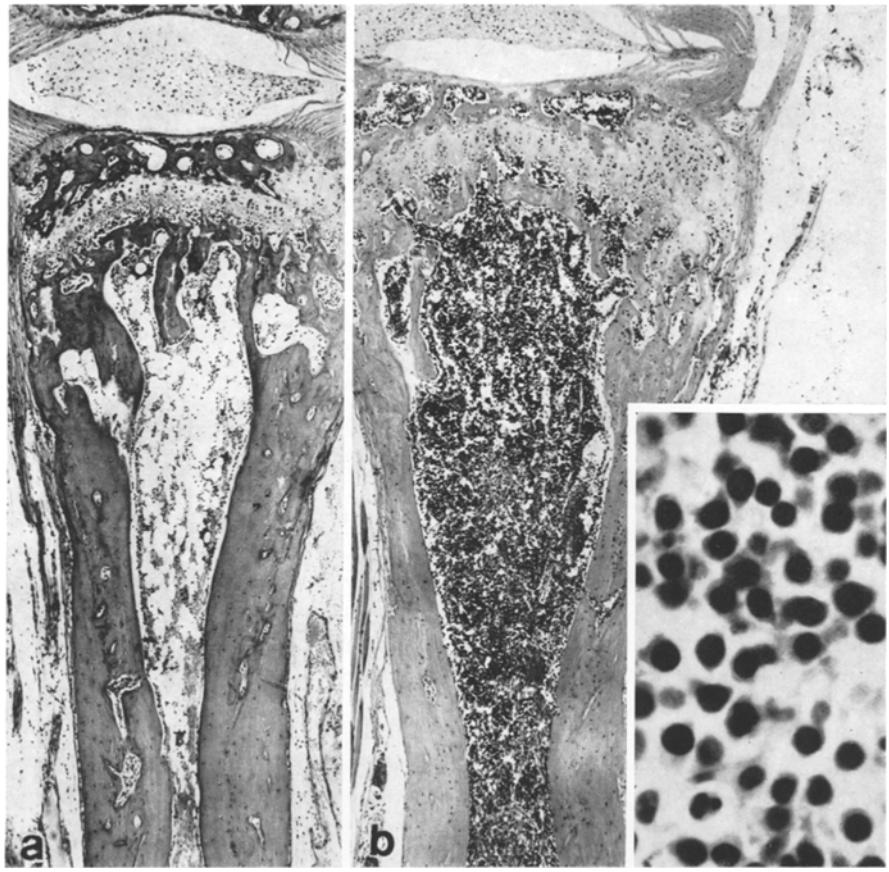

Fig. 4a and b. Longitudinal sections through tail vertebrae. (a) Control mouse: nucleated cells are few and mainly restricted to the periphery of the marrow cavity. (b) Splenectomized mouse exposed to CO continuously for 42 days: the marrow is uniformly highly cellular and contains predominantly erythroblasts (inset). H and E. a and b × 50 magnification; inset × 700 magnification

increase in the relative heart weight. Splenectomy alone appeared to produce a slight increase in the relative heart weight, both in CO-exposed and air-breathing mice, which may be seen in the last three pairs of columns of Figure 6. The differences were, however, statistically insignificant.

Histological examination of the hearts of mice, killed after 10 or 42 days of exposure to CO showed severe congestion and dilatation of blood vessels, and degeneration of the muscle fibre nuclei compared with normal organs. The mean muscle fibre diameters in left and right ventricles of normal hearts were 14.5 and 14.0 μm, respectively. In mice exposed to CO for 42 days the mean thickness of fibres in the left ventricle increased by 7.3%, and in the right ventricle by 16.7%. However, these differences were not statistically significant ($P = 0.145$ and 0.055, respectively). There were no measurable differences after 10 days' exposure.

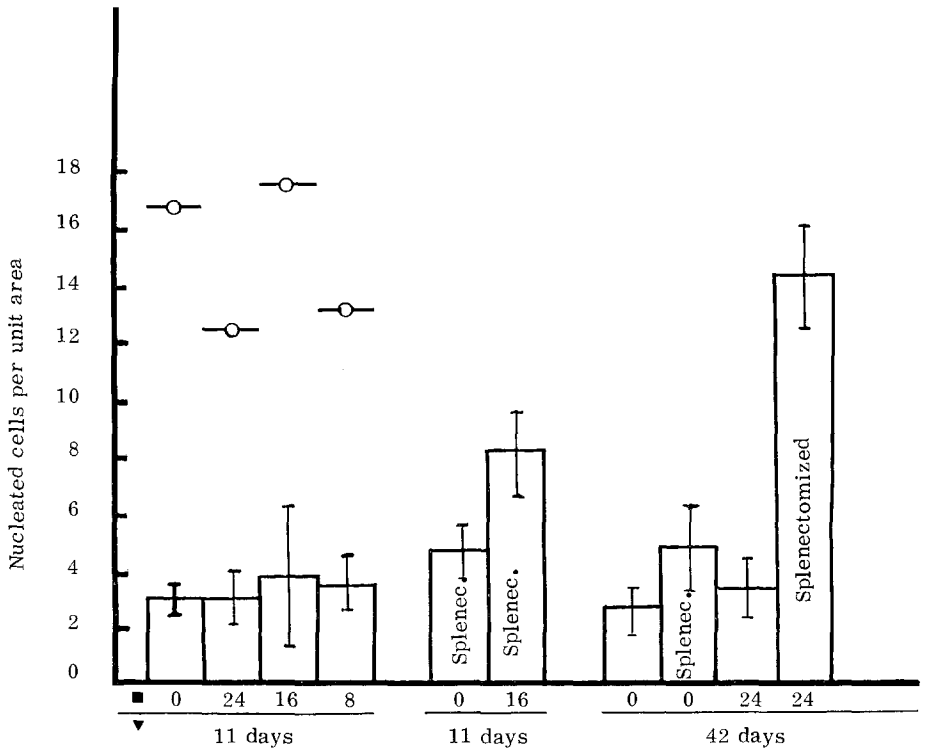

Fig. 5. Nucleated cells in tail bone marrow, expressed as number per unit area. Figures along abscissa as in Figure 3. –○– Corresponding number of cells in marrow of sternum. Each group of columns represents a separate experiment

The biochemical investigations of hearts revealed that the moisture content, protein-nitrogen and hydroxyproline content per g wet weight were the same in controls and CO-exposed mice, the differences being within the limits of experimental error.

During the course of these experiments we observed that whereas about 30% of the normal mice died within the first 42 days of CO-treatment, no splenectomized mice died. A long-term experiment to investigate the survival times and causes of death was subsequently performed, in which 12 non-splenectomized and 11 splenectomized mice were kept in CO until they died. The non-splenectomized mice all died between the 26th and 66th days, with a mean survival time 47 days; the splenectomized animals survived for 35 to 181 days, with a mean survival time of 101 days. All the non-splenectomized and 7 of the splenectomized mice died of massive peritoneal or pleural haemorrhages. In the four longest-surviving splenectomized mice, however, no haemorrhage was found, but signs of congestion, serous effusion in pleural and peritoneal cavities and wasting were present. It was noticed that deaths increased in frequency after the mice had been handled, when changing their cages, and several other mice died during the night-time. Macroscopic and histological examination showed that the site of fatal abdominal

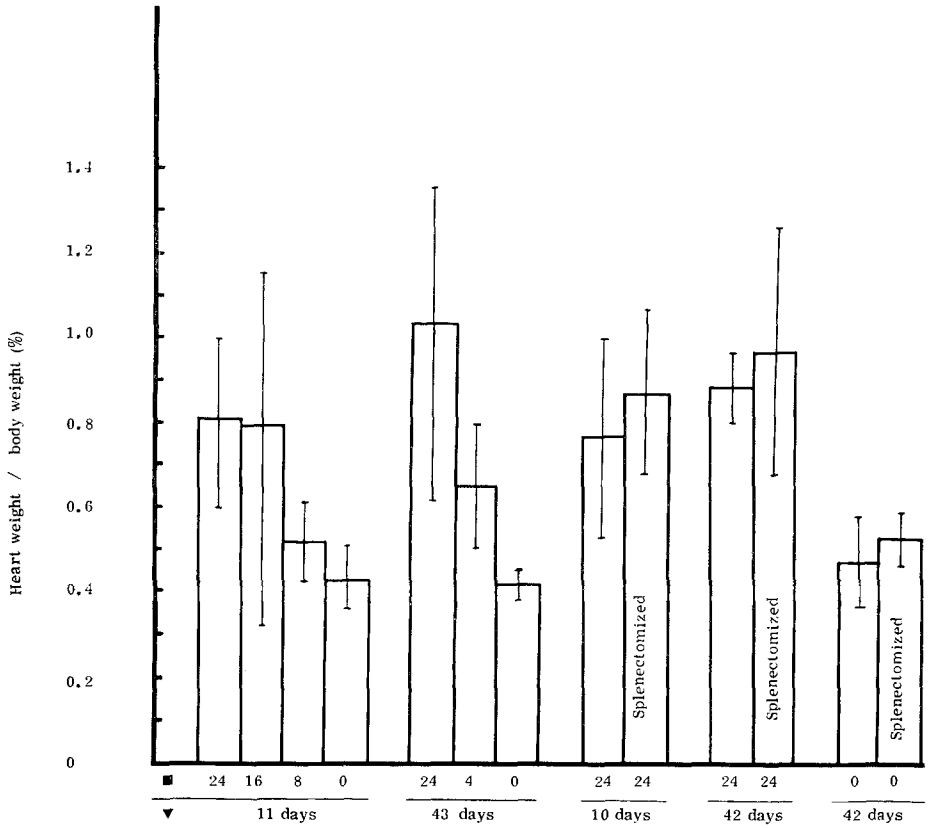

Fig. 6. Heart weights expressed as percentages of body weight. Figures along horizontal axis as in Figure 3

bleeding was the retroperitoneal space, and generally it resulted in the formation of haematomas 1–1.5 cm in diameter. The haemorrhages were fresh, without underlying inflammatory changes or thrombi in the vessels. Two sections showed either aneurysm or disruption of the wall of the aorta. No normal mice, surviving 8 months or longer, died of internal haemorrhages or showed aneurysm of the aorta. Petechiae were not found either on serosal surfaces or in the skin of any of the mice examined. Fatty tissue depletion was found in all CO-exposed mice.

Focal infiltrates of poorly differentiated cells, some of which had features of erythroblasts, were present in organs of all 6 splenectomized mice that died after 93–181 days of exposure to CO. These infiltrates were seen, in order of decreasing frequency and intensity, in the liver, kidneys, lungs, lymph nodes, adventitia of aorta, fatty tissue, endocardium and pericardium (Fig. 7a, b). In those mice which survived for 181 days the infiltrating cells had prominent hyperchromatic nuclei. It was also noticed that vascular endothelia were transformed into undifferentiated cells (Fig. 8). In addition, old and partially calcified thrombi were found in the atrial auricles in three of these mice.

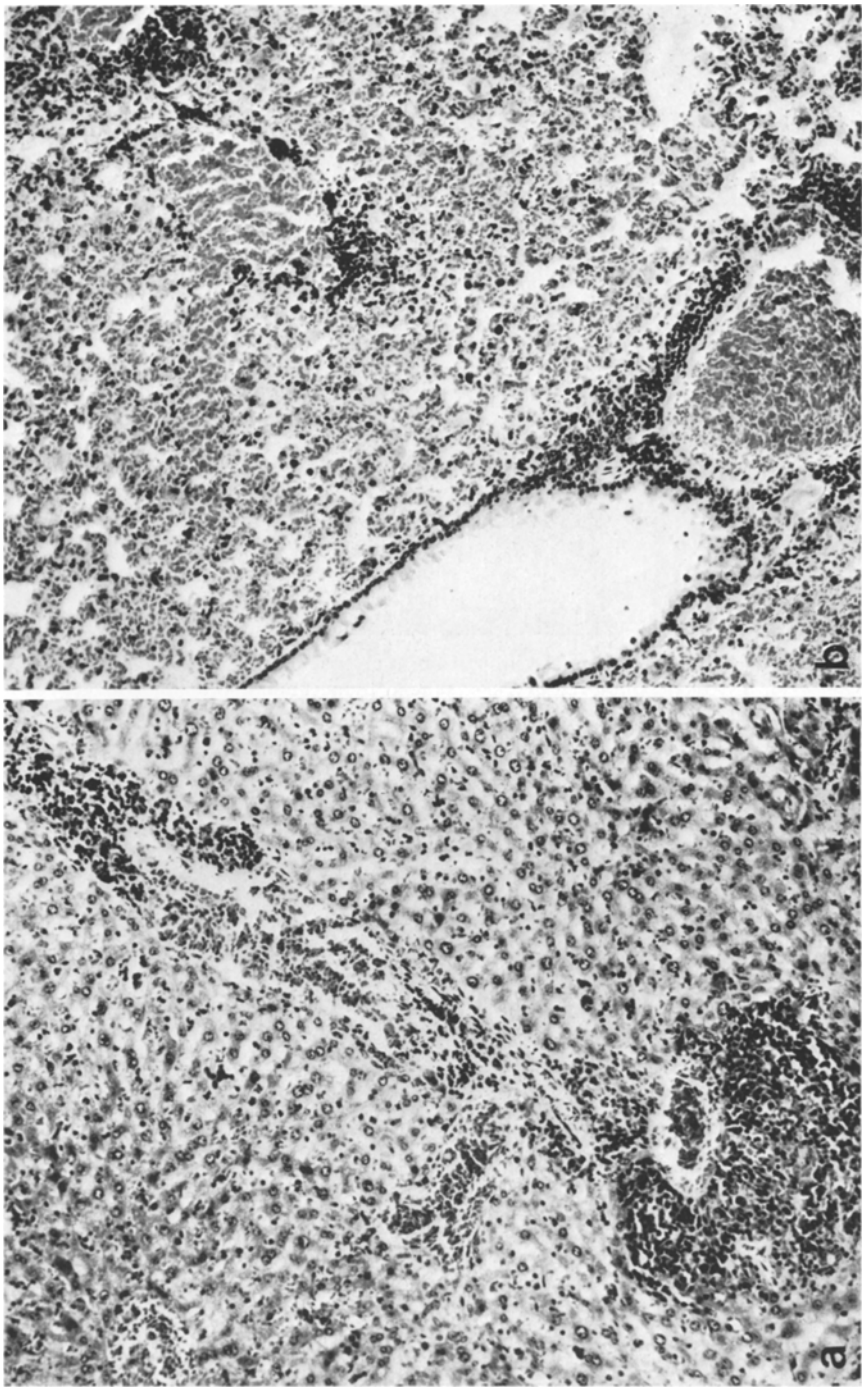

Fig. 7a and b. Sections of liver (A) and lung (B) from a splenectomized mouse exposed to CO continuously for 181 days. Multiple infiltrates of undifferentiated cells are present around vessels and bronchi. H and E., ×130 magnification

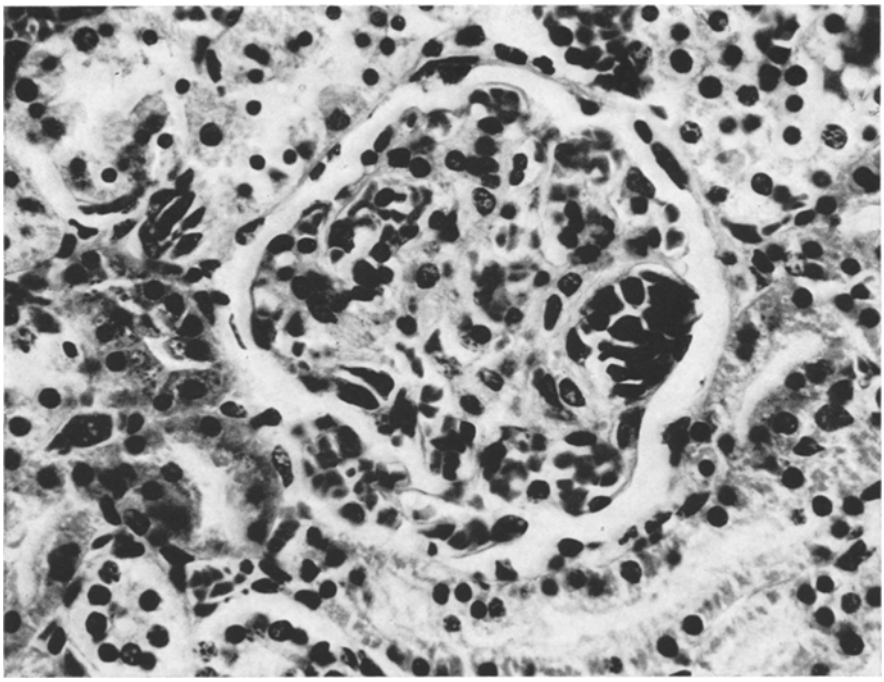

Fig. 8. Section through glomerulus of kidney from a splenectomized mouse exposed to CO continuously for 181 days. One capillary is expanded by accumulated anaplastic cells with hyperchromatic nuclei. Cells of the same type are scattered throughout the glomerulus. H and E., × 540 magnification

Discussion

By gradually increasing the concentration of CO during the first 10 days of exposure it was possible for mice to become acclimatized to 0.24% CO, and to survive in it for several weeks or months, despite blood carboxyhaemoglobin levels of about 74%. The adaptive process was manifested by a rise in haemoglobin and haematocrit levels, and the release of many reticulocytes. There was also a very marked increase in the blood volume, heart weight and spleen weight, as well as other changes associated with increased erythropoiesis.

Splenectomy generally reduced the strength of this haemopoietic response: significantly fewer reticulocytes were released into the blood and the rise in haemoglobin and haematocrit levels was consistently less in splenectomized than in non-splenectomized animals. Such an effect was predictable, since in the mouse the spleen functions as a haemopoietic organ (Aggio and García, 1969; Aggio, 1973; de Kastner and Cardeman, 1974) and this function increases in hypoxic conditions (Bozzini et al., 1970). Even without exposure to CO, splenectomy produced a temporary reduction in the haemoglobin and haematocrit levels. However, this was probably only due to the loss of blood during the operation, since bleeding alone also produced this effect, often to a greater degree.

The importance of the spleen in the adaptation of mice to CO was also evidenced by increases in both the weight of this organ and the number of

immature haemopoietic cells in the red pulp and sinusoids. Most of these cells were of the erythroblastic type. Bozzini et al. observed a similar increase in erythroid cells and iron uptake in spleens of mice subjected to bleeding and hypobaric hypoxia. Red cells in the sinusoids, on the other hand, were reduced in number relative to normal spleens. This excludes congestion as the main factor producing spleen enlargement, and is in disagreement with the view of Theodore et al. (1971) who attributed the weight increase to vascular engorgement.

The removal of the spleen did not ablate the erythropoietic part of the response to CO-hypoxia, but only reduced its intensity. Bozzini et al. considered that the erythropoietic activity of the bone marrow was normally maximal and attributed the response of splenectomized mice to hypoxia to the appearance of erythroid cells in the liver and lymph nodes. However, we only detected such extra-medullary erythropoiesis in mice kept in CO for periods in excess of 42 days, whereas a response was evident within a few days. We therefore cannot accept the assumption of Bozzini et al. and are of the opinion that the bone marrow itself may increase its output of red cells in at least three different ways. Initially, red cells are released sooner than normal, in splenectomized as well as in non-splenectomized mice, as reticulocytes. Secondly, there was some evidence of a shift in the balance between erythropoiesis and leucopoiesis. While erythrocytes increased considerably in number in the peripheral blood, both in normal and splenectomized mice, white cells had fallen slightly after 41 days' exposure to CO. In the bone marrow polymorphonuclear leukocytes were reduced, and erythroblastic and other undifferentiated cells were increased in number. Finally, there is an extension of active erythropoiesis in the tail vertebrae, the bone marrow of which is normally not highly active. In splenectomized mice exposed to CO for 42 days the tail marrow became as highly cellular as that in the lumbar vertebrae or sternum. Extra-medullary erythropoiesis appeared to develop only during the subsequent period, as infiltrates of erythroblast-like and poorly differentiated cells. By day 93 these were present in all mice examined, and it could not be excluded that in the later stages they were leukaemic, particularly erythroleukaemic, in nature. Infiltrates found in mice which survived in CO for 181 days contained cells which were anaplastic in appearance. If this is the case, it may be of considerable importance.

Exposure of rats to 0.05% CO for 24 h has been shown by Koob et al. (1974) to produce a significant decrease in their food and water intake and weight gain, which they suggested was due to a direct anorexic effect on the central nervous system. Our results are in general agreement with theirs, except that we observed a recommencement of body growth after about 14 days in CO. This corresponded to the attainment of levels of haemoglobin and haematocrit very much above normal, as well as considerable enlargement of the heart. However, even with this degree of adaptation it is doubtful if the full effects of the CO were offset. Therefore the anorexic effect is either of relatively short duration or else other factors, such as the extra metabolic requirements involved in the haemodynamic and haemopoietic responses, particularly in the period of most rapid adaptation, may also suppress growth. Even though the subsequent increase in body weight was near-normal, the general metabolism of CO-exposed mice was altered, as indicated by the apparent total depletion of all fatty tissue. Apart from causing

a slight initial weight loss, splenectomy did not affect the pattern of weight changes in CO.

The phenomenon of heart enlargement as a response to CO has been observed by many workers (Campbell, 1933; Theodore et al., 1971; Eckardt et al., 1972; Adams et al., 1973; Thomsen and Kjeldsen, 1974; Penney et al., 1974a, b, c). The concentrations which we used were very much above the threshold of 0.02% CO found by Penney et al. (1974a) to produce heart enlargement in rats. The principal factor contributing to heart enlargement in normal mice in CO is presumably the increased heart rate which is stimulated by the reduced oxygenation of the partially CO-saturated cardiac blood supply. Increased heart rate and arterial blood pressure during CO-inhalation have been reported by Ayres et al. (1970) and Adams et al. (1973) in humans and dogs, although their experiments were limited to less than 1 h. However, the polycythaemia which follows the haemopoietic response may also contribute indirectly to cardiac enlargement. Primarily, polycythaemia causes an increase in the viscosity of the blood which, by slowing the blood circulation, increases the load on the heart. A secondary effect of polycythaemia may be a compensatory increase in the plasma volume, as was indicated by the considerable increase in the blood volume. Results shown in Figure 6 (last three pairs of columns) indicate that despite the higher haematocrit values in non-splenectomized CO-exposed mice, their hearts were slightly smaller than those of corresponding splenectomized animals, thus suggesting that polycythaemia probably plays a minor role in heart enlargement.

It was also noted that the degree of enlargement of the heart was proportional to the length of daily exposure to CO: only continuous exposure produced the full effect. This may indicate a direct causal link between stimulation and heart enlargement. By contrast, near-maximum haemoglobin and haematocrit responses were elicited by exposure times as short as 8 h/day. Since bone marrow erythropoiesis is known to be mediated by erythropoietin, which has a half-life in the plasma of approximately 10 h (Stohlman, 1959), the probable explanation is that this hormone reaches sufficiently high concentrations during the hypoxic phase to remain active during the air-breathing period.

The chemical analyses excluded several factors, namely oedema, deposition of collagen or build-up of non-protein constituents, as explanations for heart enlargement during CO-exposure. The changes were, therefore, probably in the size or number of heart muscle fibres. However, the increases in fibre thickness were both statistically insignificant, even after 42 days of exposure, and also mathematically inadequate to account for the almost 100% increase in heart mass. We therefore postulate that the greater part of the increase in heart weight is due to an increase in the number of muscle fibres, as a result of proliferation (hyperplasia), and that hypertrophy is of secondary importance.

The question of hyperplasia of myocardial fibres is controversial. Some investigators deny its occurrence, maintaining that enlargement of the myocardial mass is only possible by hypertrophy of the fibres (Aschoff, 1936; Turek et al., 1972). Turek et al. measured muscle fibres in rats subjected to hypobaric hypoxia and found only hypertrophy of fibres. However, Lowe and Bate (1948a, b) produced evidence for hyperplasia of myocardial fibres in hypertensive humans who died from non-cardiac causes. Penney et al. (1974c) also accepted the

possibility of hyperplasia. This controversy may perhaps be explained by different responses to different kinds of stimulation. From the ratios of DNA to RNA in the heart muscle of rats, Martinez Seeber et al. (1974) concluded that enlargement produced by physical exercise was mainly due to muscle fibre hypertrophy, whereas that induced by nutritional anaemia was due to hyperplasia. It was of interest to note that what thickening we observed in the heart muscle fibres was greater in the right than in the left ventricle. Penney et al. (1974a) demonstrated that in rats subjected to hypobaric hypoxia the increase in the myocardial mass was also greater in the right ventricle whereas both ventricles enlarged equally during CO-exposure.

Despite the loss of splenic erythropoiesis the splenectomized mice survived in CO, paradoxically, considerably longer than normal mice. This becomes clearer when a closer consideration of the cause and mechanism of death of the CO-exposed mice is done. All non-splenectomized and 64% of splenectomized mice died of massive internal haemorrhages. The absence of petechiae on serosal surfaces or in the skin, the large size of the haemorrhages and histological findings in aortic walls suggest that they were due to the rupture of larger blood vessels, rather than to the tendency to generalized bleeding associated with impaired clotting mechanisms. We did not include platelets or other clotting factors in our study but noticed more clotting during sampling of blood from CO-treated animals. Probably of considerable importance was the distension and congestion caused by the increased volume and viscosity of the blood. It is also possible that in the hypoxic state produced by breathing CO the amounts of oxygen diffusing into the thicker arterial walls had fallen below the critical level in places, resulting in degeneration and necrosis. The weakened wall then developed an aneurysm and finally ruptured, particularly when the animals became active. The observation that deaths were most frequent immediately after handling the mice is consistent with this. It is likely that in splenectomized mice the blood viscosity was lower than in non-splenectomized animals, in which the highest haematocrit values were recorded. Thus their risk of haemorrhage should also have been lower.

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