The Mechanism of Damage to the Bone Marrow in Systemic Poisoning with Mustard Gas

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(Received 21 April 1947)

One of the most important features in recent investigations on mustard gas ($\beta\beta'$ -dichlorodiethyl sulphide, referred to as *H* throughout the paper) is the focusing of attention on the systemic effects of this poison. The general appreciation in this country of the importance of this type of effect was caused by a series of reports by Cameron and his collaborators (Cameron, 1941*a, b*; Cameron, Short & Foss, 1942; Courtice & Cameron, 1942) in which, besides giving a summary of earlier observations, they described a number of pathological experiments of their own from which it becomes clear that *H* when applied in small doses to the skin of rats (5–10 mg.) or rabbits (10–15 mg.) causes, apart from its local effects, definite widespread changes throughout the organism. These changes are very severe in fatal cases, and they are here undoubtedly the only reason for death, because local symptoms are in these experiments either insignificant or (where the *H* is given by injection) non-existent.

According to Cameron, changes in the bone marrow are amongst the most specific and striking effects of this systemic poisoning. Here the white cell system is mainly affected: there is a degeneration of polynuclear leucocytes and myelocytes leading to a total disappearance of these cells; the bone marrow is fatty and oedematous; the megakaryocytes show dispersion and pyknosis of their nuclei; they are vacuolar and swollen and their boundaries are frayed out. Alterations in the red cell system are absent or much less marked and they usually occur at a much later stage. Leucopenia after an initial leucocytosis is probably mainly a result of these changes. The blood leucocytes also show marked degenerative changes. Among other systemic effects are an acute gastritis and changes in lymphoid tissue. The latter consist in depletion of lymphocytes, and in degeneration and focal necrosis in the spleen, lymph glands and lymphoid tissue.

The following experiments were undertaken in order to throw light on the biochemical mechanism underlying the systemic effects. In view of the indication from previous work on local lesions that enzymes connected with carbohydrate metabolism (phosphokinases) are especially affected by *H* (Dixon & Needham, 1946), the investigation of the glucose utilization of suitably chosen organs seemed most promising. We therefore studied the anaerobic glycolysis of the bone marrow of poisoned animals; and in

order to assess the specificity of the bone marrow damage we made similar observations in the spleen and brain. The damaged tissues were also examined histologically, and in a few observations in the early stages of poisoning we attempted to discover whether the histological or the metabolic changes could first be recognized. We were interested in this time relationship because, if there had been a significant interval between the first appearances of these two types of change, it might have thrown light on the pathogenesis of the lesions.

It seems highly probable that the widely distributed systemic changes are caused by some toxic substance, circulating most probably in the blood stream. We therefore tried to examine the properties of this compound by (1) testing the effect of serum from *H*-poisoned animals on the metabolism of normal marrow *in vitro*; (2) by comparing *in vitro* the effect of *H* on metabolism of marrow with that of *H* previously hydrolyzed in serum; (3) by observing the effects of excluding from the circulation part of the animal's marrow during the first period (up to 1 hr.) after injection with *H*; (4) by testing the possibility of the temporary storage of unchanged *H* in fat, and its subsequent release.

EXPERIMENTAL METHODS

Preparation of rabbit marrow for manometric experiments. The rabbit was stunned by a blow on the head and killed by bleeding. Immediately after death the femora and tibiae were dissected out, and both ends were cut off. In some cases the humeri were also used. The marrow was forced out of the bone by a blast of air into Ringer solution, and was then squeezed through coarse muslin and shaken. On standing, the fatty substance quickly rose to the surface and was pipetted off. The marrow cells were then spun down and resuspended in Ringer solution for the manometric experiments. By using a suspension of cells thus freed from most of the fat, it was possible to obtain quite uniform samples; the cells from the marrow of one animal were usually made up to about 8 ml. Duplicates of 0.5 ml. were used for the estimations of N content, and 2 or 3 ml. samples for the experiment. About 30 mg. dry wt./pot was a convenient amount for the glycolysis experiments; larger amounts were used for respiration measurements. When the manometric experiment was to be done in serum, the Ringer suspension, after removal of the samples for nitrogen estimations, was recentrifuged, and the cells were

made up to the same volume with serum. The Ringer solution had the following composition: 0.8% NaCl, 116 ml.; 1.0% KCl, 3 ml.; 1.12% CaCl_2 , 2 ml. In the bicarbonate-Ringer, 20 ml. of the NaCl solution were replaced by 20 ml. of 1.26% NaHCO_3 solution.

Preparation of spleen. Since the preparation of satisfactory tissue slices from the spleen is difficult, a suspension of spleen cells was used. This was obtained by cutting the spleen into small pieces suspended in Ringer solution, and squeezing them through coarse muslin. The suspension was centrifuged, and further treatment was just as for the marrow.

Preparation of serum. Fresh rabbit blood was collected and defibrinated at once by shaking with glass beads. After straining through glass wool, it was centrifuged and the serum was pipetted off. It was then ready for use in the glycolytic experiments, but for respiration experiments it was necessary to remove bicarbonate and to prepare 'neutralized' serum, as described by Warren (1934). This was done by adding 0.1N-HCl till the pH had fallen to 6.6, evacuating till no more CO_2 was evolved, and adding 0.4M-phosphate buffer, pH 7.4, to make a final phosphate concentration of M/150. The final pH should be 7.4; this was checked, and when occasionally necessary, was adjusted by addition of 0.1N-NaOH.

For the glycolysis experiments done in serum it was necessary to take account of the retention of CO_2 by the serum. The simplest method is to calculate new constants for the Barcroft apparatus, in which the effect of retention is included. The retention curves of the sera, giving the relation between the CO_2 retained and the pH, are first obtained as described by Brekke & Dixon (1937). The new constants are then calculated by the following method, for which we are indebted to Dr Dixon:

Let an amount of acid be produced such that the manometric reading (h) increases by 1 mm.

Let B_1 be the bicarbonate content before the change in $\mu\text{l. (N.T.P.)}$ in the amount of serum used; B_2 the bicarbonate content after the change; C_1 the initial CO_2 pressure in mm. manometric fluid; C_2 the final CO_2 pressure in mm. manometric fluid, and c the correction factor for the manometer, i.e.

$$\frac{\text{change of reading}}{\text{change of pressure in experimental vessel}}$$

(see Dixon, 1943). Under our conditions this could be taken as 1, without appreciable error.

Let K be the constant of the manometer for CO_2 when there is no retention and K^* be the desired constant taking account of retention.

$$\text{Then } B_1 - B_2 = K, \quad C_2 - C_1 = c.$$

Then the change of pH corresponding to an increase of 1 mm. in the reading is given by

$$\Delta \text{pH} = \log (B_1 - K) / B_1 + \log C_1 / (c + C_1)$$

from the Henderson-Hasselbalch equation.

From the retention curve, which is almost linear, the retention R corresponding to ΔpH is determined. The new constant is given by $K^* = K + R$. The retention correction varies slightly with the reading, but under our conditions the error produced by taking K^* as constant does not exceed 4%.

Preparation of marrow fat. We wished to know the effect upon normal cell glycolysis of adding the marrow fat from

poisoned animals. It was found that the fatty layer separated off as described above contains nitrogen and shows glycolytic activity. In order to free the fat from metabolizing cells, the fatty layer was mixed with an equal volume of distilled water, allowed to separate out again and removed; the process was repeated. After it had been pipetted off for the third time, the fatty layer was centrifuged, the fat removed and excess water drained away on filter paper. In this way, plasmolysis of cells should be obtained and any enzymically active cell-free extract should be washed away. The fat prepared in this way did indeed show very slight or no metabolic activity.

Estimation methods. Anaerobic glycolysis was estimated by measuring the CO_2 produced in serum or in bicarbonate-Ringer solution in an atmosphere of 95% N_2 /5% CO_2 , in the Barcroft apparatus. Respiration was measured in the Barcroft apparatus in 'neutralized' serum, in an atmosphere of oxygen, in presence of KOH papers; a few experiments were done in the Dixon-Keilin apparatus in bicarbonate-Ringer in an atmosphere of 95% O_2 /5% CO_2 . In all this work, glucose was present, unless otherwise stated, in the serum or bicarbonate-Ringer solution, in concentration of 0.2%. In the glycolysis experiments with serum, bicarbonate solution was used in the left-hand pot, but in the respiration experiments more satisfactory results were obtained if the neutralized serum was used on both sides of the manometer.

The results for both glycolysis and respiration are expressed in $\mu\text{l. CO}_2$ produced or O_2 absorbed/mg. fat-free dry wt./hr. ($Q_g^{\text{N}_2}$; Q_{O_2}). The fat-free dry weight was obtained by estimating N in measured portion of the suspension and multiplying by the factor 6.85 (Warren, 1934) for marrow, and 6.25 for spleen. The incineration was carried out in conc. H_2SO_4 , with a mixture of SeO_2 , K_2SO_4 and CuSO_4 as catalyst.

Poisoning of animals. In all experiments except those specially mentioned, the dose of H for rabbits was 5 mg./kg. In most of the work, the pure redistilled H was dissolved immediately before use in thiodiglycol, to obtain a solution containing 50 mg./ml. The required amount of this was injected into the ear vein. In a few later experiments the H was dissolved in triacetin.

Histological methods. Material was fixed in Zenker formol, and paraffin sections were stained by Ehrlich's haematoxylin and eosin method and by the method of Barrett (1944) which gives results similar to those of Maximow's haematoxylin-eosin-azure method. In the rat experiments, Leishman-stained smears as well as sections were used, and in preparation of the sections the bones were decalcified by Custer's (1933) method.

EXPERIMENTAL RESULTS

Marrow

Glycolysis of marrow. The anaerobic glycolysis of normal marrow was first determined both in bicarbonate-Ringer solution and in normal rabbit serum. The results agreed with those of Warren (1934, 1940) in showing greater activity in serum (Table 1). In the absence of glucose there was practically no glycolysis.

Table 1. *Anaerobic glycolysis of normal marrow*

$Q_g^{N_2}$ of normal marrow	
In bicarbonate-Ringer	6.0, 6.5, 6.0
In serum	9.0, 14.0, 10.0, 9.5, 10.6, 10.5, 9.0

The activity in serum of marrow from poisoned animals, killed at different times after injection of *H*, is shown in Table 2 (see also Fig. 1).

Table 2. *Anaerobic glycolysis of poisoned marrow*

Time after injection of <i>H</i> (hr.)	$Q_g^{N_2}$ of marrow	Degree of histological abnormality
0	10.3*	—
4.5	7.6	Slight
10.5	7.3	Moderate
12	3.9	Moderate
15.5	2.7, 3.4	Moderate
21	<3.0	(Not examined)
48	<1	Severe
72 (in bicarbonate)	<2	Moderately severe
18 (by skin application)	2.5	Moderate

* Mean value for normal animals.

Fig. 1. Glycolysis of bone marrow from normal and *H*-poisoned animal in normal serum. 1, marrow from normal animal; 2, marrow from animal poisoned 12 hr. previously.

The progressive diminution in $Q_g^{N_2}$ with time after injection is seen; even as early as 4.5 hr. after injection, the value was slightly lower than any normal obtained, and by 12–15 hr. after injection the decrease is very marked.

One experiment was done to ascertain if similar metabolic changes accompany the histological damage in the bone marrow after application of *H*

to the skin. *H* (55 mg. in 0.25 ml.) was painted on a shaved area of about 15 cm.². The results are given at the end of Table 2.

Histological evidence of damage was already present, though only slight, after 4–5 hr.; and it is interesting that it was the erythroblasts not the leucocytes which showed the most conspicuous abnormality at the early stage. The nucleus of the affected cells showed a kind of hydropic degeneration. While retaining its sharp outline and often several nodes of chromatin around its periphery, it was considerably swollen and completely empty-looking except for one or rarely two eosinophilic granules which seemed to be floating free in its interior. These granules probably represented nucleoli but were more strongly eosinophilic than the nucleoli of normal cells of the same kind. It was often difficult to decide whether cells affected in this way were erythroblasts or plasma cells: at first they were thought mostly to be plasma cells but it now seems certain that many if not all were nucleated red cells. A few myelocytes and other cells showed this hydropic nuclear degeneration. Granular leucocytes were still abundant but some showed karyorrhexis.

The histological changes seen in the later stages of damage for the most part agreed well with previous descriptions (Cameron, 1941*b*). In all except the very earliest stages, the cells of the granular leucocytes series were affected more than those of the erythrocyte series. The lack of parallelism between the hydropic nuclear degeneration and the other changes suggests that a different mechanism was involved. The most striking feature of marrows in which the damage is assessed in Table 2 as 'moderate' usually was a conspicuous decrease in the number of mature granular leucocytes, associated with an increase, apparently not merely relative, in the number of early myelocytes with scanty granules. These myelocytes were often unhealthy-looking and unusually large, but not rarely they were seen to be undergoing mitosis. Damage assessed as severe approached complete aplasia.

Respiration of marrow. The values found for respiration of normal marrow and those found after injection of *H* are given in Table 3. It will be seen that after about 14 hr., the respiration had fallen to only 30–50 % of the normal.

Table 3. *Respiration of marrow*

Q_{O_2}	
Normal marrow	2.0, 1.6, 1.2, 1.6, 0.9, 2.0
Poisoned marrow (hr. after injection)	
15	0.45
12.5	0.47
14	0.84
15	0.60

Effect of H and of H hydrolyzed in serum on marrow glycolysis in vitro. The suspension of marrow cells in Ringer solution was divided into three equal parts, which were centrifuged separately.

(a) The cells were suspended in 2 ml. normal serum as usual; 0.025 ml. ethylene glycol monoethyl ether (e.g.e.) was tipped in on each side after equilibration, and the glycolysis was measured.

(b) The cells were suspended in 2 ml. normal serum, and after equilibration in the Barcroft pots, 0.025 ml. 4% *H* in e.g.e. was tipped into the suspension from a Keilin tube. (Bicarbonate, 2 mg. in 0.02 ml., had previously been added to this serum, the amount being equivalent to the acid expected from hydrolysis of the *H*.) An equal volume of e.g.e. was tipped into the left-hand pot. The glycolysis was measured.

(c) The cells were suspended in 2 ml. serum previously treated with *H* in the following way: 4 ml. serum (+4 mg. bicarbonate in 0.04 ml. water) were placed in the Barcroft apparatus in an atmosphere of 95% N_2 + 5% CO_2 at 38° and equilibrated as usual; 0.05 ml. 4% *H* in e.g.e. was tipped in and the reaction was allowed to continue till no more gas was evolved. This took about 45 min. The marrow cells were then suspended in 2 ml. of this serum and their glycolysis was measured.

Fig. 2. Glycolysis of bone marrow under influence of *H* and of decomposed *H* in serum. 1, normal serum; 2, serum in which *H* has been decomposed; 3, serum containing *H*.

Fig. 2 shows the results. When *H* was added giving a concentration of 0.0026M, inhibition was immediate, and amounted to about 60%. After reaction of *H* in serum the same concentration had no effect.

The effect of serum from poisoned animals on the glycolysis of normal marrow. In these experiments

the suspension of marrow cells in Ringer solution was divided into equal portions, which were centrifuged separately. The samples were made up with equal volumes of normal and of *H*-serum respectively. The poisoned sera used were: (a) obtained 4.5 and 21 hr. respectively after intravenous injection of the usual dose of 5 mg. of *H*/kg. (a separate rabbit being used for each experiment). There was no effect on the glycolysis of the cells suspended in them (see Fig. 3). (b) Obtained 2 and 1 hr. respectively after the injection of a very large dose (25 mg./kg.) a separate rabbit being used for each experiment. The rabbits were in both cases extremely ill, and would probably not have lived many minutes longer.

Fig. 3. Glycolysis of bone marrow in normal serum and in serum of poisoned animals. 1, serum of animal poisoned 4.5 hr. previously with *H*; 2, serum of normal animal.

In the first experiment with serum (b), obtained 2 hr. after the injection of *H*, an apparent 60% inhibition of glycolysis of marrow suspended in it was observed. It was found, however, that both bicarbonate content and pH of this serum were extremely low, the former equivalent only to 123 μ l. CO_2 /ml. of serum, and the latter 6.84. The glycolysis measurement was therefore repeated after addition of the calculated amount of sodium bicarbonate to bring the content back to normal.

In this medium the marrow glycolysis showed no significant deviation from normal (Q_0^N in normal serum 10.5, in poisoned serum 9.0).

The experiment was repeated, using *H* serum 1 hr. after injection, the bicarbonate content having been adjusted as before. In this experiment, in order to give better opportunity for any toxic effect to become manifest, the marrow cells were suspended, before the glycolysis determination, in a comparatively large volume of serum, without glucose and in air. The freshly prepared marrow suspension in Ringer solution was divided into two parts and these were centrifuged. The cells were suspended in 8 ml. of normal and of *H* serum respectively and shaken at 38° in Barcroft pots, with the taps open for 80 min. The contents of the pots were then centrifuged, the cells were suspended in 1.25 ml. of the appropriate serum (now containing glucose) and the glycolysis was measured.

The $Q_{O_2}^N$ was 4.4 in the normal and 4.6 in the poisoned serum. (The low value for normal marrow is usual after much prolonged treatment.)

We have thus found no effect on the marrow glycolysis of serum from animals poisoned with *H*, apart from the effect due to low bicarbonate content of the serum after a very large dose.

The effect of suspending the marrow cells in the medium obtained by haemolyzing red blood cells from an animal 15.5 hr. after poisoning was also tested, and again the result was negative.

The effect of fat from marrow of poisoned animals on marrow glycolysis. It is known that *H* is more stable in a fatty than in an aqueous environment. If one could assume that *H*, after having been stored in the fat depots of the body is slowly released, the major difficulties in the explanation of the mechanism of the systemic action of *H* would be solved. The long latent period would become understandable and the postulation of a toxic intermediate would become unnecessary.

In order to examine the toxic properties of fat isolated from bone marrow of rabbits poisoned with *H* at different times previously we incubated for 45 min. 2 ml. of normal bone marrow suspension with 500 mg. of fat obtained from poisoned animals. A control was done each time with 2 ml.

of the same normal marrow cell suspension and 500 mg. normal fat. After the incubation the anaerobic glycolysis was determined. For this purpose the contents of the Barcroft pots were made up to 3 ml. with bicarbonate solution containing glucose (to give the usual concentrations of bicarbonate and glucose). We usually allowed the glycolysis to go on for several hours in order to detect a possible quicker falling off at a late stage in the poisoned fat-normal marrow mixture as compared with the control. In all cases but one the glycolytic action of the fat involved was investigated, and found to be very low or nil. This glycolysis, when it occurs, is most probably to be attributed to cells or cell extracts which have escaped the washing.

From the results (Table 4), it is clear that the poisoned fat had no inhibitory influence on the glycolysis of normal bone marrow. It is possible that by the procedure of washing the *H* dissolved in the fat is removed. It seems likely, however, that if a considerable amount of *H* were really present in the fat before the washing, some would reveal itself afterwards during the long incubation with marrow (up to 3 hr.). Further, in Exp. 4, washing was restricted to a minimum. Here the marrow was collected dry (not into Ringer solution) and the fat was washed as rapidly as possible. The result was that fat so prepared kept some activity but inhibition did not occur.

Spleen

Glycolysis of spleen suspensions from normal and poisoned animals. These measurements were all done in bicarbonate (Table 5). After 21 hr., at a time when marrow glycolysis had fallen by about 70 %, spleen glycolysis was unchanged. Even after 48 hr. it had only fallen by about 50 %.

In the last experiment (48 hr.) histological examination showed considerable abnormality of the spleen. There was a conspicuous deficiency of lymphocytes in the Malpighian corpuscles, the absent lymphocytes being replaced by large pale cells with

Table 4. *Effect of fat from poisoned animals in marrow glycolysis*

Rabbit*		Total glycolysis in μ l. CO ₂							Glycolysis for marrow alone in μ l. CO ₂ /flask/hr. after interaction with		
Wt. (kg.)	Killed after injection (hr.)	Fat Marrow...	Normal Normal	Poisoned Normal	Normal —	Poisoned —	— Normal	No fat	Normal fat	Poisoned fat	
2.90	2.5		134	142	Considered negligible	0	—	—	134	142	
2.35	5.0		110	104	0	0	—	—	110	104	
2.45	13.0		—	258	Considered negligible		226	226	—	227	
3.60	5.5		160	165	40	34.6	—	—	120	130.4†	

Care was taken that in every experiment dealing with the effect of different fats on the same suspension of normal marrow cells, exactly the same amount of the latter was added to each flask, so that the rates of glycolysis obtained/flask could be compared directly.

* In each case 5 mg. *H*/kg. was injected intravenously.

† In this experiment the washing was restricted to a minimum.

oval nuclei, presumably reticulum cells. Polymorphonuclear leucocytes were fairly numerous but showed much evidence of destruction, karyorrhexis being common and free nuclear fragments abundant.

Table 5. *Glycolysis of spleen suspensions*

	$Q_{\text{O}_2}^{\text{N}}$	
Normal spleen	5.5, 7.3, 5.8, 7.8	
Poisoned spleen		
(hr. after injection)	10	5.5
	12	8.0
	15	6.5
	21	6.5
	48	3.5

In the experiment previously mentioned, in which *H* was painted on the skin instead of injected, and in which marrow glycolysis had fallen by about 75 %, the spleen glycolysis was almost normal, $Q_{\text{O}_2}^{\text{N}}$ 5.1. Histological examination in this case showed only moderate abnormality; there was some reduction in the number of lymphocytes in the Malpighian bodies, but polymorphonuclear leucocytes were abundant and mostly appeared healthy.

Respiration of spleen suspensions from normal and poisoned animals. The respiration of spleen suspensions in serum was also inhibited after *H* poisoning. The results (Table 6) suggest that in the spleen the inhibition of respiration may occur earlier than the inhibition of glycolysis.

Table 6. *Respiration of spleen suspensions*

	Q_{O_2}	
Normal spleen	1.7, 1.9	
Poisoned spleen		
(hr. after injection)	21	1.0
	24	0.7
	48	0

Brain

Anaerobic glycolysis and respiration of brain slices from normal and poisoned rats. Adult female rats, weighing between 180 and 240 g., were painted with a dose of 3 mg. *H* in 0.03 ml. e.g.e. on the shaved skin of the back. Since

these experiments were more prolonged than most of those with rabbits, it was important that both control and experimental animals should receive the same amount of nourishment. As the *H*-painted animals scarcely eat, all the animals were given three subcutaneous injections daily of 3 ml. 50 % glucose. After 48 hr., the animals were killed by bleeding and slices were made from the cerebral cortex. Anaerobic glycolysis was measured in the usual way in bicarbonate-Ringer solution, respiration in the Barcroft apparatus in Ringer solution containing 0.0066 M-phosphate, pH 7.4, or in the Dixon-Keilin apparatus in bicarbonate-Ringer. The results for normal and poisoned animals are given in Table 7. The dry weight was obtained by removing the slices at the end of the experiment, washing them, drying at 110° and weighing.

It was found that, in sharp contrast to the marrow, after as much as 48 hr. from the time of poisoning, neither anaerobic glycolysis, nor respiration nor respiratory quotient was significantly altered.

The effect on bone marrow excluded from the circulation after injection of H

The simplest explanation for the activity of *H* would be that it is absorbed into the blood stream from the place of application, and carried unchanged with the blood to the bone marrow, which is poisoned as in the *in vitro* experiments previously described. The flaw in this conception is that it takes several hours for the intoxication of the marrow *in vivo* to make itself detectable by our methods, while *in vitro* there is a marked inhibition of glycolysis immediately after application of the poison. The reason for this difference might of course be the much higher degree of dilution in the experiments *in vivo*. In any case, one would expect with a mechanism of this kind (that is to say, with *H* acting *qualitate qua*) that the changes which condition the damage in the marrow should occur within a very limited period after injection because after that time all the free *H* will have disappeared in a blood medium, as will be shown presently.

This question was investigated by excluding a part of the marrow from the circulation for a certain

Table 7. *Anaerobic glycolysis and respiration of brain cortex slices*

		$Q_{\text{O}_2}^{\text{N}}$	
Normal brain	...	10.0, 16.0, 11.5, 12.0, 10.4, 10.0 (av. 11.6)	
Poisoned brain	...	7.0, 7.0, 13.0, 7.2, 9.8, 10.7 (av. 9.1)	
		Medium	Q_{O_2} R.Q.
Normal brain	Phosphate-Ringer	- 10.5	—
Poisoned brain	Phosphate-Ringer	- 12.0	—
Normal brain	Phosphate-Ringer (no glucose)	- 4.5	—
Poisoned brain	Phosphate-Ringer (no glucose)	- 6.4	—
Normal brain	Bicarbonate-Ringer	- 14.5	0.92
Normal brain	Bicarbonate-Ringer	- 11.0	1.0
Normal brain	Bicarbonate-Ringer	- 14.0	1.0
Poisoned brain	Bicarbonate-Ringer	- 12.9	1.09
Poisoned brain	Bicarbonate-Ringer	- 14.0	0.88

period after injection of the poison, to see if this would save this marrow in contrast to other parts of the marrow not excluded. The length of this period

measured by the acid production, is about 60 % complete in 20 min. and about 95 % complete in 1 hr.

Fig. 4. Decomposition of *H* in heparinized plasma and whole blood. 1, *H* in plasma; 2, *H* in blood.

must depend on the rate of reaction of *H* in blood, and this was measured in the Barcroft apparatus at 38° by tipping a known amount of *H* into 3 ml. of blood after equilibration, and following the CO₂ output. The curves (Fig. 4) show that the reaction, as

We used a technique similar to that described by Smith (1943) for the following experiments with rats. The abdomen was opened under ether anaesthesia and the vena cava and aorta abdominalis were clamped just below the origin of the renal vessels thus excluding the hind legs from the circulation. Then *H* in thiodiglycol was injected into the vena cava, centrally to the clamp, in a dose of 2 mg./kg. After removal of the clamp, the wound was closed. During the clamping the animal was kept warm, and the intestines were occasionally irrigated with saline at 37°. Controls were done with animals clamped for the same period, but only given the solvent. After the animals were killed, one humerus and one femur from each were examined histologically. The results are recorded in Table 8 and illustrated in Pl. 5.

In the control animals, the bone marrow in both the humerus and femur is either normal or showed a variable degree of acute inflammatory reaction presumably due to the operative treatment (see Pl. 5 C, D) but in the animals which received an injection of *H* a very different picture was seen (Pl. 5 A, B). The changes were similar to those produced by *H* in the bone marrow of rabbits, but it was more noticeable in the rats that eosinophil leucocytes were more resistant to the reaction of *H* than neutrophil leucocytes. In severely damaged marrows, when neutrophil leucocytes and indeed most other types of cell had almost completely disappeared, eosinophil leucocytes and tissue mast cells were still abundant. Despite the clamping of the abdominal aorta and vena cava for 20–60 min. after the injection of *H*, all the rats showed damage to the femoral bone marrow which was not appreciably less severe than that in the humerus. Thus the

Table 8. *Effect of exclusion of bone marrow from the circulation*

No.	Wt.	Duration of clamping (min.)	Injected with	Time elapsed before killing (hr.)	Histological damage	
					Humerus	Femur
1	168	20	TG	24	None	None
2	162	20	TG	24	None	None
3	194	20	—	24	None	None
4	209	20	2 mg. <i>H</i> /kg.	48	Severe	Severe
5	182	20	2 mg. <i>H</i> /kg.	24	Severe	Severe
6	167	20	2 mg. <i>H</i> /kg.	48	Severe	Severe
7	210	60	TG	24	None	None
8	208	60	TG	23	None	None
9	193	60	2 mg. <i>H</i> /kg.	21.5	Moderate	Moderate
10	247	60	2 mg. <i>H</i> /kg.	20	Moderate	Moderate
11	240	0	TG	72	None	None
12	233	60	TG	48	None	None
13	245	60	2 mg. <i>H</i> /kg.	48	Severe	Severe
14	182	60	2 mg. <i>H</i> /kg.	48	Severe	Severe
15	245	60	2 mg. <i>H</i> /kg.	72	Severe	Severe

TG = thiodiglycol. The *H* solutions were made up immediately before use, to contain 2 mg. *H*/ml. TG. The volume of the injection fluid used was in all cases 1 ml./kg. The rats used were all females.

poisoning of the marrow was not exclusively determined in the first hour after injection and therefore it seemed unlikely to be due to a direct action of unchanged *H* brought by the blood stream. However, the size of the dose of *H* may be important, because in another similar experiment, not recorded in Table 8, in which some of the *H* leaked from the syringe during the injection so that the effective dose was less than 2 mg./kg., the marrow in the humerus was severely damaged but that in the femur was little, if at all, affected.

These results agree with those obtained by Smith (1943) who, using small doses of mustard gas, found that bone marrow could be protected by exclusion from the circulation but using larger doses could confirm our results. Since the humerus was exposed to the action of any toxic substance in the blood both during the hour of clamping and afterwards, whereas the femur was only exposed afterwards, it is not surprising that with small doses the marrow of the humerus may be damaged while that of the femur is not. But with the dosage used by us, our experiments *in vitro* suggest that at least 95% of the *H* injected would have been hydrolyzed during the period of clamping; yet the marrow of the femur appeared as severely damaged as that of the humerus. We conclude that it is unlikely that the marrow damage was due to the direct action of unaltered *H*.

DISCUSSION

The inhibition of glycolysis in marrow, spleen and brain is not surprising, in view of what we know of the effect of *H* on the glycolytic enzymes *in vitro*, and on glycolysis in skin. The main aspects are: (a) the long delay, even greater than in skin, although *in vitro* the inhibitory effect was immediate; (b) the specificity of the effect on marrow which was much more affected than other organs in the early stages, both as regards cell damage and inhibition of glycolysis.

Concerning the mechanism of the delay, another significant finding, in the experiments involving clamping off of parts of the circulation, was that with the dosage which we employed the bloodstream appeared to be as efficient in intoxicating the tissue if it came in contact 1 hr. after the injection of *H* as immediately after injection. We have tried to test the two possible explanations for the lack of protection of the excluded marrow in the case of *H*:

(a) The long continued circulation of a stable toxic product formed from *H*, which might for example, be a compound of *H* with protein. We obtained no inhibitory effect on normal marrow glycolysis by serum or haemolyzed red cells taken from animals after *H* poisoning. This cannot, however, be taken as ruling out the presence of an in-

hibitory substance in the bloodstream. In the experiments *in vitro*, the normal marrow cells can only be exposed to the *H* serum for a comparatively short time (3–4 hr.) and to a comparatively small volume. *In vivo*, the marrow is exposed for a much longer time before marked inhibition appears; and possibly as the blood circulates through the marrow, the toxic substance in it, even though present in low concentration, may react with some essential constituent in the marrow, so that finally the active amount of this constituent is reduced below some limiting value, and inhibition of glycolysis is manifested.

(b) The accumulation of unchanged *H* in fat, from which it is later slowly released into the aqueous phase. Here again, our negative results with fat prepared from poisoned marrow could not be taken as final, since it was possible, though not likely, that the fat was detoxicated during the preparation as carried out. Later experiments, however, using radioactive *H* showed that there is no specific accumulation of radioactive sulphur in bone marrow fat (Boursnell, Cohen, Dixon, Francis, Greville, Needham & Wormald, 1946).

The interpretation of the results showing inhibition by *H* of the marrow glycolysis and respiration is complicated by the fact that the relative amounts of white and red cells in the marrow change. The proportion of white cells becomes less with time after the poisoning and it is known from the work of Warren (1934) that the glycolytic activity of the white cells is greater than that of the red cells, while their respiratory activity is less. Clearly, a fall in the relative amount of white cells would (even in the absence of any direct effect of *H* on the cell metabolism) lead to a decrease in glycolysis and an increase in respiration, when these are expressed/mg. dry weight. What we have in fact found is a decrease in both glycolysis and respiration. The fall in respiration must be due to a direct effect upon the cells. We are inclined to think that the fall in glycolysis is also in part due to direct effect on the cells, especially the white cells, since it is marked at a time when the actual disappearance of leucocytes has not gone far, although the cells are abnormal. In the later stages, when the proportion of red cells is very high, the decrease in glycolysis is probably the result of two factors, the disappearance of the very active white cells, and the inhibition which may affect the red cells. In these late stages, the inhibition of respiration is probably chiefly due to the effect on the red cells.

SUMMARY

1. After intravenous injection of a lethal dose of mustard gas (*H*), the anaerobic glycolysis and the respiration of rabbit bone marrow were inhibited.

A, $\times 70$, marrow from femur of rat killed 48 hr. after injection of *H* into the clamped inferior vena cava, the clamp being released 60 min. after the injection. Ehrlich's haematoxylin and eosin. B, $\times 70$, control marrow from femur of normal rat. C, $\times 70$, marrow from humerus of same rat as A. D, $\times 70$, control marrow from humerus of normal rat.

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This inhibition was progressive, being just obvious after 4 hr. and very marked after 24 hr., and it ran parallel with the cell damage observed histologically.

2. Anaerobic glycolysis was also inhibited in the rabbit spleen, but only after a much longer time (48 hr.). At this late stage, histological damage was present.

3. No inhibition of anaerobic glycolysis or respiration was observed in brain slices from rats 48 hr. after poisoning.

4. We were not able to find any inhibitory effect on the anaerobic glycolysis of normal marrow cells by serum obtained from rabbits poisoned with the usual dose of *H*.

5. Neither haemolyzed red cells from a poisoned rabbit nor fat obtained from the marrow of such animals, had any effect on the anaerobic glycolysis of normal marrow cells.

6. Addition of *H* (0.0026M) to marrow cells *in vitro* caused immediate inhibition amounting to 50 %. No effect was observed with the same amount of *H* after reaction with serum.

7. The acid production from *H* in serum and in whole blood took about 45 min. for completion at 38°; 85 % was produced in 30 min.

8. Excluding part of the bone marrow of rats from the circulation for 1 hr. after injection of a lethal dose of *H* (2 mg./kg.) did not protect the marrow from the cell damage caused by *H*, as determined histologically.

This work was carried out for the Ministry of Supply in 1942-3, as part of the work of an Extramural Research Team under the direction of Dr M. Dixon, and was contained in a report to the Ministry dated 1944. We are indebted to the Chief Scientist Ministry of Supply for permission to publish this paper. We would also express our gratitude to Dr M. Dixon for his constant advice and interest.

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* Information available on application to Ministry of Supply, London.

Precipitation of Steroid Ketones with Digitonin

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(Received 15 April 1947)

It is frequently held that a steroid is precipitable by digitonin by virtue of a free 3-hydroxyl group of the β -configuration (formula (I)). There are in fact at least four known exceptions to this rule, namely, *allopregnan-3(β)-ol-20-one acetate*, *allopregnane-3:20-dione* (Butenandt & Mamoli, 1935), ' α '-oestradiol and its 3-benzoyl derivative (Wintersteiner, 1937). In the course of some work on the metabolism of cholest-4-en-3-one, it was discovered that this substance also is precipitable by digitonin. The following is a brief account of this reaction applied to some steroid ketones.

METHODS

The ketones, together with four parts by weight of digitonin, were dissolved with heating, in 90 % ethanol-water. After

at least 24 hr. at 18-20°, the digitonides were collected, washed with cold 90 % ethanol-water, and ether, dried at c. 70° and decomposed by a single pyridine-ether treatment in the usual way. The ketones, recovered by evaporation of the washed ether, were crystallized from dilute ethanol. Table 1 shows the weights of digitonide and of crystalline recovered ketone. The amounts used were 50 mg. of ketone and 200 mg. of digitonin in all cases except that of progesterone, when 25 mg. of ketone and 100 mg. of digitonin were used. Recovered ketones were identified by mixed melting-point determinations.

The digitonin used in the above experiments did not itself crystallize out in 24 hr. at 18-20°, although it sometimes did so on longer standing. Unlike the precipitates of digitonides (except, perhaps, that from progesterone) the digitonin crystals themselves were readily soluble in cold water.