Some Aspects of Phosphorus Metabolism in Bone Marrow

By C. LUTWAK-MANN

Biochemical Laboratory, University of Cambridge

(Received 7 December 1950)

The chemical composition and the metabolism of bone marrow have been recently studied by several investigators, including Warren (1934, 1940); Schultze (1941); Goldinger, Lipton & Guzman Barron (1947); Needham, Cohen & Barrett (1947); Davidson, Leslie & White (1947, 1948, 1950); Evans & Bird (1949); Dietz (1949) and Dietz & Steinberg (1949).

This paper deals with certain aspects of phosphorus metabolism in bone marrow, and summarizes results obtained in the course of the last 4 years (Lutwak-Mann, 1947*a, b*, 1949, 1951; Lutwak-Mann & Gunz, 1949), based on experimental material from some 60 rabbits and well over 1500 rats. It was necessary to use relatively large numbers of animals because of the variations observed in the phosphorus content of the bone marrow of normal animals. These were due to species differences as well as, within one species, to not inconsiderable fluctuations between members of different breeds. To reduce the influence of these and other factors, such as age and sex, young adults only were used, the rabbits being litter mates of the same breed, and the rats males of a highly inbred laboratory stock.

The phosphorus content of rabbit and rat bone marrow was first investigated in normal untreated animals; next, the effect was examined upon the phosphorus content of extraneous factors such as changes in the composition of food leading to undernourishment or vitamin deficiency; furthermore, phosphorus metabolism was followed in the bone marrow of animals exposed to the action of myelotoxic agents such as mustard gas and X-rays. In addition, *in vitro* experiments were carried out to study the phosphorus distribution under conditions of tissue autolysis.

MATERIAL AND METHODS

Material. The bone marrow was removed from the long bones (femur, tibia, humerus), after dissection of the epiphyses, by means of compressed air. The quantities of material collected in this manner varied in rabbits between 3 and 11 g. and in rats from 120 to 200 mg. fresh weight per animal. The animals were thoroughly bled after decapitation and little blood was present in the normal bone marrow; on several occasions the haemoglobin content of the marrow samples was estimated and found to correspond to blood diluted 1 in 15 to 1 in 20; it is not likely, therefore, that the

residual blood could have affected the analytical results to any appreciable degree.

Methods. Phosphorus was determined by means of the Fiske & Subbarow (1925) method. In the text below 'directly determinable P' applies to phosphate which reacted directly with molybdate, 'readily hydrolysable P' is the fraction which hydrolysed at 100° in *N*-HCl in 10 min. ($P_{10} - P_0$), and 'difficultly hydrolysable P' corresponds to the difference between total acid-soluble P and the fraction which hydrolysed in 120 min. ($P_{120} - P_{10}$). Adenosinetriphosphate (ATP) and $\text{NH}_3\text{-N}$ were determined by the method of Parnas & Lutwak-Mann (1935). Total nucleic acid, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and lipid P were estimated according to the Schmidt & Thannhauser (1946) procedure. For the estimation of acid-soluble P precautions were taken to avoid the presence of bone splinters.

Mustard gas (*H*) was routinely applied to rats (180–220 g.) as follows: 0.1 ml. of 4% (v/v) solution of *H* in ethylene-glycol monoethyl ether was painted on the plucked skin (roughly 1 in. square) on the back of an animal anaesthetized with ether, left on for 5 min. and washed away with ethanol, soap and water. Under such conditions a certain amount of *H* evaporates, and it is probable that less than half the amount applied actually penetrates the rat skin. Nevertheless, when these conditions were strictly adhered to, a remarkably reproducible picture developed in response to the treatment. The animals stopped eating and drinking 24–36 hr. after the start of the experiment; they had severe gastro-intestinal disturbances from the 2nd or 3rd day onwards, and showed all the typical symptoms of systemic *H* poisoning between the 3rd and 4th day, with chromatocoryrrhoea and prostration leading to coma; there was severe leucopenia at that time. The animals which survived the 4th–6th days were likely to live on for some weeks, but frequently late deaths occurred at 3–4 weeks.

Mustard sulphoxide (*HO*), which unlike *H* has unreactive chlorine atoms and is non-vesicant, was administered by subcutaneous injection. The symptoms depended upon the dose: with amounts corresponding roughly to those of the cutaneous *H* application, i.e. 0.01–0.02 mg./g., the animals were practically unaffected except for a slight loss of weight; 0.05 mg./g. produced some toxic effects, and 0.1 mg./g. was nearly as toxic as the routine dose of *H*. But even then, although the animals showed severe gastro-intestinal disturbances, they were never quite so ill as those which had been treated with *H*; in particular the nervous symptoms were much less pronounced. Leucopenia of moderate to severe degree was evident after the administration of 0.05–0.1 mg./g.

Dimethyldithiocarbamate (DMT), a substance originally introduced by Cameron & Rydon (1944) as an antidote in systemic *H* poisoning, was administered subcutaneously in

doses of 0.1–0.2 mg./g.; there were no symptoms except for a passing somnolence soon after the injection. Diethylthiocarbamate (DET), urethane and thiourea were given by subcutaneous injection in the above quantities and had, when given alone, no visible ill effects upon the rats.

A combination of small, by themselves ineffectual, doses of *HO* (0.01–0.02 mg./g.) with DMT was invariably followed by severe toxic symptoms which resembled those characteristic of either *HO* in high dosage (0.1 mg./g.) or of *H* itself. Even when DMT was injected not simultaneously with, but 7–10 hr. after, a small dose of *HO*, the clinical symptoms of *H* poisoning could still be produced.

A combination of small amounts of *HO* with thiourea also led to symptoms of some severity; the addition of urethane to a low dose of *HO* did not affect the general condition of the rats, but, judging from results obtained with nucleic acid P in the bone marrow (Table 8), there was a distinct effect upon this tissue.

Irradiation experiments were carried out with rabbits and rats; some of the results were described earlier (Lutwak-Mann & Gunz, 1949). The animals received single doses of röntgen radiation to the body as a whole; a dose of 1000 r. was given to 2.5–3.5 kg. rabbits, and 500 r. to 180–240 g. rats. The irradiations were carried out by the Staff of the Department of Radiotherapeutics, University of Cambridge, by kind permission of Prof. J. S. Mitchell, with a 220 keV. Maximar machine, fitted with an Al filter only; a dose rate of 130 r./min. was applied in a 180 sq.cm. field. Rabbits which received a dose of 1000 r. usually showed loss of weight for about 2 weeks, a few also developing intestinal symptoms; none died during the 2–3 weeks' experimental period. Rats varied in their response to irradiation; some appeared relatively slightly affected, whereas others succumbed within a few days with severe symptoms of widespread haemorrhage and diarrhoea. The surviving animals, about half the number of those exposed to radiation, showed loss of hair and greying, and a poorer increase in weight than the controls; their spermatozoa when examined at 8–10 weeks after exposure were largely immotile and disintegrated.

A small number of experiments was carried out in which bone marrow was exposed *in vitro* to large doses of X-rays (10,000 and 20,000 r., at a dose rate of 1000 r./min., and 250,000 r. in the course of 20 min.); the bone marrow was thinly spread on a flat watch glass and irradiated, while the control sample was kept for the same length of time; both samples were then used for P analysis, without further incubation.

For the nutritional experiments control rats were maintained on a full synthetic diet which consisted of 23 parts of light white casein, 40 of rice starch, 17 of cane sugar, 15 of arachis oil, 5 of salt mixture and 2.5 of cod liver oil, supplemented with 7.5 parts of dried yeast; the vitamin B-deficient rats received the same food without the yeast supplement. Vitamin B₁ deficiency was produced by feeding the following diet: cane sugar 60 parts, light white casein 20, arachis oil 15, salt mixture 5, cod liver oil 2.5, supplemented by 12 parts of autoclaved Marmite for the deficient, and the same quantity of untreated Marmite for the control animals. Vitamin B₁ used in these experiments was crystalline aneurin (Roche Products Ltd.); 'vitamin B complex', kindly supplied by Dr E. Kodicek, Nutrition Laboratory, University of Cambridge, was made up as described by Kodicek & Carpenter (1950); vitamin B₁₂ was 'Cytamen' (Glaxo); folic acid was 'Folvite' (Lederle).

RESULTS

Phosphorus compounds in the bone marrow of normal rabbits and rats

The distribution of various phosphorus fractions in fresh bone marrow is represented in Table 1; it will be seen that in rabbit as well as in rat bone marrow the level of directly determinable P showed fluctuations; these are thought to be largely the outcome of enzymic activity of the bone marrow and caused by the unavoidable delay in the collection of the material. A greater amount of readily hydrolysable P was found than corresponded to ATP pyrophosphate calculated on the basis of the ATP amino N determination; this discrepancy, of varying extent, was presumably due to the presence in bone marrow of acid-labile P compounds other than ATP. The variations encountered in total acid-soluble P in the bone marrow of rabbits may be considered real, as it was found that this particular phosphorus fraction was relatively stable during incubation at room temperature (Table 3); but in rat bone marrow total acid-soluble P rose markedly during incubation (Table 4) so that the variations in its content may be at least partly due to this circumstance.

Table 1. *Distribution of phosphorus compounds in bone marrow of rabbits and rats*

(Average values calculated from fifteen determinations, except in the case of rat nucleic acid P which is based on forty determinations. P₀=phosphorus determined as phosphate which reacts directly with ammonium molybdate; P₁₀ and P₁₂₀=phosphorus which appears as orthophosphate after 10 and 120 min. hydrolysis with N-HCl; P_{tot.}=total phosphate after incineration; ATP amino N=nitrogen of the adenine amino group of ATP determined as NH₃ after enzymic deamination; P_{lipid}=phospholipin phosphorus; P_{nuc.}=total nucleic acid phosphorus.)

	Acid-soluble P (mg./100 g.)				ATP amino N (mg./100 g.)	P _{lipid} (mg./100 g.)	P _{nuc.} (mg./100 g.)
	(P ₀)	(P ₁₀ - P ₀)	(P _{tot.} - P ₁₂₀)	(P _{tot.})			
Rabbit							
Average	5.7	7.7	10.6	40	0.7	25	77
Range	3.8–8.3	3.6–11.0	8.0–12.6	32–48	0.4–1.6	27–37	48–123
Rat							
Average	35	11	28	95	—	56	250
Range	29–42	7–14	24–36	72–115	—	47–60	220–300

Lipid P showed fairly constant values in rabbit, and somewhat higher ones in rat, bone marrow. The difference was greatest in the nucleic acid P content of rabbit and rat bone marrow. In rabbits chosen at random, values for nucleic acid P differed from each other very considerably; better agreement was found in litter mates (Table 2). On the other hand, very good reproducibility existed with regard to nucleic acid P in members of the laboratory stock colony of hooded rats, where there appeared to be some minor sex difference which, however, was eliminated by the exclusive use of young adult males. The concentration of nucleic acid P in rat bone marrow was on the average three times higher than in rabbits.

Table 2. *Nucleic acid phosphorus in the bone marrow of litter mate rabbits*

Litter	Nucleic acid (mg. P/100 g.)
A	113, 123, 98, 120
B	57, 62, 60
C	72, 79
D	88, 95

Changes in the distribution of bone marrow phosphorus in the course of incubation in vitro

To study the behaviour of acid-soluble P during incubation of bone marrow, the following experiments were set up. Rabbit bone marrow from several animals was collected, with the least delay, on muslin fixed on a tared beaker; the material was gently squeezed through muslin, weighed and dispersed in 5–8 vol. of water or saline. With the aid of a blunt pipette samples were removed from the suspension, sedimentation of particles being prevented by frequent stirring and shaking.

For initial values a sample was at once deproteinized with trichloroacetic acid; others were incubated with some toluene at 24° for 4 hr., either in air or in nitrogen, and then deproteinized. From the protein-free filtrate samples were taken for the estimation of directly determinable and total P, as well as of ATP and ammonia N; the rest was

brought to pH 8.3 and precipitated with barium acetate + 5 vol. of ethanol containing ammonia; after removal of barium, this fraction was used for the determination of directly determinable, readily and difficultly hydrolysable P. The results of a representative experiment of this kind are in Table 3. It will be seen that in rabbit bone marrow only a small proportion of acid-soluble P was not barium-precipitable; total acid-soluble P under these incubation conditions increased only slightly; however, there was an appreciable accumulation of inorganic P in the course of incubation, particularly in the absence of oxygen. Readily hydrolysable P increased aerobically, but was diminished in the absence of oxygen, whereas ATP amino N was well maintained in air, and even in nitrogen somewhat less than half the initial amount was still present at the end of the incubation; as in fresh bone marrow, more readily hydrolysable P was found than could be accounted for by the ATP pyrophosphate P. The decrease in the difficultly hydrolysable P fraction, due to the enzymic activity of the bone marrow, was presumably responsible for the rise in inorganic P. The ability of bone marrow to dephosphorylate various added phosphate esters was demonstrated in separate experiments and referred to earlier (Lutwak-Mann, 1949). The above processes concerned with the phosphorus metabolism of the bone marrow were accompanied by production of ammonia, irrespective of the presence of oxygen, in amounts which exceeded several times the content of preformed ATP amino N.

The behaviour of acid-soluble as well as nucleic acid P was also examined in rat bone marrow. It was found that at the end of a few hours incubation at 38° of 150–200 mg. of bone marrow in 2 ml. of water or buffer solution with some toluene, there was an appreciable increase in total acid-soluble P and a fall in nucleic acid P; estimations of DNA and RNA showed that during a 5 hr. incubation the DNA/RNA ratio increased to 3.4 from the initial value of 1.75, indicating that the breakdown of RNA was chiefly responsible for the loss in total nucleic acid content.

Table 3. *Changes in the content of acid-soluble phosphorus compounds and ammonia during aerobic and anaerobic incubation of rabbit bone marrow*

(Incubation 4 hr. at 24° in presence of toluene. Deproteinization with trichloroacetic acid. From the filtrate samples taken for determinations of P_0 , $P_{tot.}$ and NH_3 ; in another sample ATP amino N determined by enzymic deamination; the rest of the trichloroacetic acid filtrate precipitated with barium acetate at pH 8.3 in presence of 5 vol. ethanol. For symbols see Table 1.)

	P in trichloroacetic acid filtrate (mg./100 g.)		ATP (mg./100 g.)		P fractions in Ba precipitate (mg./100 g.)				Free ammonia (mg. NH ₃ -N/100 g.)
	P ₀	P _{tot.}	Estimated NH ₃ -N	Calculated labile P	P ₀	(P ₁₀ - P ₀)	(P _{tot.} - P ₁₂₀)	P _{tot.}	
Before incubation	11.2	34.0	0.76	3.37	11.5	5.0	10.6	29.3	1.30
After aerobic incubation	15.2	35.6	0.61	2.70	16.5	7.6	4.8	29.8	3.41
After anaerobic incubation	26.0	36.2	0.32	1.42	24.6	2.1	2.2	32.0	3.58

To establish how far these findings were specific for bone marrow, similar experiments were carried out with suspensions of rat spleen. The results for bone marrow and spleen are in Table 4. They show that there was a fall in the content of nucleic acid P in the spleen, but it was less marked than in the bone marrow; acid-soluble P increased in both tissues during incubation; the rather high initial level of acid-soluble P in the rat bone marrow in this particular instance was probably the result of delay in the collection of sufficient experimental material. Since phospholipin P was not determined in these experiments, it was not possible to decide what proportion of the acid-soluble P increment was due to lipid breakdown and how much was derived from nucleic acids. Considerable amounts of ammonia accumulated during the incubation of rat bone marrow, and somewhat less with rat spleen. When suspensions of bone marrow (or spleen) were heated for 10 min. at 85°, or precipitated with trichloroacetic acid, and then incubated, the processes described here were largely abolished. In rabbit

bone marrow incubated at 38° there was also a loss in nucleic acid P, but to a much smaller extent than in rat bone marrow.

Several experiments were carried out to study some details of the enzymic breakdown of nucleic acid P in rat bone marrow. It was found that whereas the reaction as assessed by the disappearance of total nucleic acid P was largely independent of pH, DNA appeared to be less resistant at low than at high pH values; on the other hand, even more RNA disappeared in an alkaline than in an acid medium. The decomposition of nucleic acid proceeded readily in suspensions in water, and in phosphate, veronal and glycine buffers (Table 5). Ammonia was also determined in these experiments; somewhat more of it was produced under conditions which enhanced the breakdown of RNA, that is at higher pH values.

The *in vitro* exposure of rat bone marrow to doses of röntgen radiation ranging from 10,000 to 250,000 r. did not affect the content of nucleic acid P. The addition *in vitro* of H, HO, or HO together with

Table 4. *Changes in the content of acid-soluble phosphorus, nucleic acid phosphorus and ammonia during incubation of rat bone marrow and spleen*

(Suspensions of fresh bone marrow or ground spleen, 150–200 mg. tissue in 2 ml. water, incubated in presence of toluene, and deproteinized with trichloroacetic acid. Total acid-soluble P ($P_{ac. sol.}$) and NH_3 determined in the trichloroacetic filtrate. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) determined as P in the trichloroacetic acid precipitate.)

	Incubation time (hr.)	P compounds (mg. P/100 g.)			DNA RNA	NH_3 (mg. N/100 g.)
		$P_{ac. sol.}$	DNA	RNA		
Bone marrow	0	129	153	87	1.75	3.8
	3	210	120	45	2.66	28.2
	5	232	109	32	3.40	43.0
Spleen	0	78	100	66	1.51	3.5
	1	95	95	58	1.67	10.5
	3.5	131	87	50	1.74	16.3
	5	162	72	40	1.80	22.0

Table 5. *Effect of pH on the breakdown of deoxyribonucleic and ribonucleic acid and the formation of ammonia in rat bone marrow incubated in vitro*

(Rat bone marrow, 150–200 mg., suspended in 2 ml. water or 0.06M-buffer, control deproteinized at once, the other samples incubated at 38° in the presence of toluene, and then deproteinized with trichloroacetic acid; ammonia determined in the protein-free extract, DNA and RNA in the trichloroacetic acid precipitate.)

Exp. no.	Medium	pH	Incubation time (hr.)	DNA (mg. P/100 g.)	RNA (mg. P/100 g.)	NH_3 (mg. N/100 g.)
1	—	—	0	139	81	10
	Water	6.0	2	114	50	22
	Veronal-acetate	4.9	2	125	44	23
		6.9	2	136	41	26
		8.0	2	138	36	29
2	—	—	0	160	80	11
	Veronal-acetate	6.9	3.5	130	45	35
		8.0	3.5	147	40	41
	Phosphate	7.0	3.5	149	34	41
		8.0	3.5	153	33	48
3	—	—	0	165	80	—
	Glycine-HCl	1.9	1	122	67	—
	Glycine-NaOH	12.0	1	149	54	—

DMT, in a final concentration of 0.005M to aqueous suspensions of incubated bone marrow, did not alter appreciably the rate of breakdown of nucleic acid P as compared with samples incubated without these additions.

The dependence of the nucleic acid phosphorus content in rat bone marrow upon variations in the composition of food

As previously stated, the content of nucleic acid P in the bone marrow of rats belonging to an inbred stock varied little in animals of the same age and sex. In connexion with research on the action of myelotoxic agents which frequently cause deterioration in the general condition of the animal as well as diminished food intake, it was essential to establish to what extent the bone marrow content of nucleic acid P depended upon quantitative and qualitative alterations of the stock diet.

Rats weighing 140–180 g. were maintained for 4–5 weeks on diets deficient in B group vitamins, or in vitamin B₁ only; control animals were subdivided into groups some of which were pair fed with the deficient rats, and others which were fed *ad lib*. In addition, several of the deficient as well as the non-deficient rats were treated parenterally for 2 weeks with 'vitamin B complex' or aneurin, and also with vitamin B₁₂ or folic acid, during which period they were either pair fed with deficient untreated animals, or allowed to consume food without restriction. Some of the results were reported earlier (Lutwak-

Mann, 1951). In addition to the bone marrow the secretory function of the male accessory glands of reproduction, which had previously been shown to be very sensitive to nutritional deficiency (Lutwak-Mann & Mann, 1950), was also examined in all these experimental animals. The findings have been summarized briefly (Lutwak-Mann & Mann, 1951).

Table 6 shows the effect of dietary changes on the nucleic acid P content of rat bone marrow. General vitamin B deficiency, or B₁ deficiency, reduced considerably the content of this phosphate fraction in the bone marrow; but a quantitative restriction of non-deficient food by pair feeding also led to a distinct decrease. As regards treatment with vitamins, in animals receiving the full diet an excess of aneurin was without effect; the condition in the deficient animals could be improved with vitamins of the B complex or with aneurin only if at the same time there was no quantitative limitation of food intake. The results in Table 6, which relate to deficient rats treated with vitamin B₁₂ or folic acid, were carried out on pair-fed rats, to study the ability of these substances to stimulate rat bone marrow irrespective of food consumption. However, treatment of rats with vitamin B₁₂ or folic acid, unlike B complex or aneurin, failed to improve the general health of the animals and had no beneficial effect so far as the content of nucleic acid P in the bone marrow was concerned.

The changes brought about by inadequate feeding were not irreversible: when the deficient animals

Table 6. *Effect of diet on the nucleic acid phosphorus content of rat bone marrow*

(Groups nos. 1, 5 and 10 comprise ten animals each, the rest four animals each.)

Group no.	Diet	Nucleic acid (mg. P/100 g.)			
		DNA		RNA	
		Mean	S.D.	Mean	S.D.
1	Full synthetic diet	166.1	9.34	85.5	6.95
2	Full synthetic diet + 200 µg. aneurin daily	166.5	3.54	82.5	4.95
3	Full synthetic diet restricted by pair feeding with vitamin B-deficient rats	140.5	9.19	70.5	4.95
4	Full synthetic diet restricted by pair feeding + 100 µg. aneurin daily	139.0	8.94	73.5	0.71
5	Vitamin B-deficient diet	106.1	23.24	58.1	7.18
6	Vitamin B-deficient + 100 µg. aneurin daily; unrestricted food intake	140.8	12.34	97.5	9.98
7	Vitamin B-deficient + 0.4 ml. 'B-complex'; unrestricted food intake	147.0	12.73	88.5	7.78
8	Vitamin B-deficient + 10 µg. vitamin B ₁₂ daily; pair fed	122.7	30.00	69.3	22.28
9	Vitamin B-deficient + 0.5 mg. folic acid daily; pair fed	101.0	18.39	53.5	11.18
10	Vitamin B ₁ -deficient diet	85.2	20.50	52.8	14.58
11	Vitamin B ₁ -deficient + 100 µg. aneurin daily; unrestricted food intake	123.0	16.24	67.5	17.68

were placed on full diet for about 3 weeks a gradual recovery took place, and normal or near normal values of nucleic acid P were found in the bone marrow. It may be added here that whereas it was possible to restore completely the secretory function in the accessory glands of deficient animals, regardless of food consumption, by treatment with testosterone propionate or chorionic gonadotrophin, these hormones had no effect whatsoever upon the content of nucleic acid P in the bone marrow.

The effect of mustard gas (H) and mustard sulphoxide (HO) on the phosphorus content of bone marrow

In continuation of earlier studies which dealt mainly with systemic effects of *H* and *HO* poisoning with particular reference to the glycolysis of bone marrow and gastric mucosa (Lutwak-Mann, 1947*b*), experiments were carried out to examine the phosphorus content in the bone marrow of animals treated with these substances.

Table 7. *Effect of mustard gas (H), mustard sulphoxide (HO), and HO in combination with dimethyldithiocarbamate (DMT), on acid-soluble and nucleic acid phosphorus content of rat bone marrow*

(Rats, 180–200 g. body weight, on full synthetic diet. Injected doses are expressed in mg./g. body weight; *H* applied cutaneously, 0.1 ml. 4% solution in ethylene glycol monoethyl ether for 5 min.; *HO*, 0.02 mg., and DMT, 0.2 mg., subcutaneously. The values for total acid-soluble P ($P_{ac. sol.}$) and nucleic acid P ($P_{nucl.}$) are average figures from three to four rats, expressed as mg. P/100 g. bone marrow. Ranges in brackets.)

Time after injection (days)	Untreated controls		<i>H</i>		<i>HO</i>		<i>HO</i> + DMT		DMT	
	$P_{ac. sol.}$	$P_{nucl.}$	$P_{ac. sol.}$	$P_{nucl.}$	$P_{ac. sol.}$	$P_{nucl.}$	$P_{ac. sol.}$	$P_{nucl.}$	$P_{ac. sol.}$	$P_{nucl.}$
1	93 (72–110)	249 (238–250)	68* (58–70)	168* (148–180)	—	—	—	—	—	—
2	—	—	51* (50–55)	62* (50–71)	80 (75–100)	250 (228–260)	53* (49–68)	75* (62–80)	81 (70–97)	222 (200–241)
5	—	—	—	—	—	250 (220–258)	96† (80–118)	178† (120–188)	—	—
7	—	—	102 (98–110)	225 (178–240)	—	—	105 (90–128)	233 (180–245)	—	—
10	87 (78–115)	256 (242–262)	94 (88–97)	222 (196–250)	70 (50–108)	262 (250–268)	130† (125–136)	234† (210–247)	78 (67–88)	239 (223–244)

* Admixture of blood in bone marrow; diarrhoea.

† Bone marrow of soft consistency but no blood.

‡ Bone marrow strikingly pale and fatty.

Table 8. *Effect of various doses of mustard sulphoxide (HO), alone and in combination with diethyldithiocarbamate (DET), urethane, and thiourea, on the content of acid-soluble and nucleic acid phosphorus in rat bone marrow, 48 hr. after administration*

(Rats, 200–220 g. body weight, on stock diet. P values are averages from two to three rats; for symbols see Table 1.)

Substances injected	Dose (mg./g. body weight)	P fractions (mg. P/100 g.)				DNA RNA
		Acid-soluble P		Nucleic acid P		
		(P ₁₀ - P ₀)	P _{tot.}	DNA	RNA	
<i>HO</i>	0.01	9.0	95	175	88	1.97
	0.05	6.3	100	106	80	1.32
	0.10	—	78*	59*	47*	1.25
<i>HO</i> + DET	0.01	4.3	61	46	39	1.18
	0.10	—	65	169	81	2.08
DET	0.10	—	65	169	81	2.08
<i>HO</i> + urethane	0.01	7.1	102	144	82	1.75
	0.10	—	100†	87†	62†	1.40
<i>HO</i> + thiourea	0.01	—	100†	87†	62†	1.40
	0.10	—	93	159	79	2.01
Urethane	0.10	10.0	81	167	83	2.01
Thiourea	0.10	—	93	159	79	2.01

* Considerable admixture of blood in bone marrow; diarrhoea.

† Slight admixture of blood in bone marrow.

Table 7 records experiments in which a comparison was made of the action upon acid-soluble and nucleic acid P in bone marrow, of roughly equivalent doses per g. body weight of *H* and *HO*, the latter being used alone and in combination with DMT, in rats which survived the treatment for 10 days. It will be seen that at 24 and 48 hr. following the cutaneous application of *H*, parallel with the development of clinical symptoms, there was a considerable fall both in acid-soluble and in nucleic acid P; a dose of 0.02 mg./g. *HO* alone was without effect, but when administered together with DMT, it provoked changes in the bone marrow analogous to those due to *H* itself. Towards the end of the 10-day experimental period the nucleic acid P values were restored to nearly the normal level; it was interesting to note a rise in acid-soluble P at this stage in the bone marrow of rats treated with *HO* + DMT. The latter by itself had little effect upon the phosphorus content of bone marrow.

Table 8 illustrates the early effects upon acid-soluble and DNA and RNA P of various doses of *HO*, and also of small doses of *HO* administered together with DET, urethane and thiourea. *HO* (0.1 mg./g.) affected the bone marrow in the same manner as the (much smaller) routine dose of *H*. DET was at least as effective as DMT, when used in combination with *HO*. Urethane alone was without effect; however, together with *HO*, it produced a slight but distinct fall in nucleic acid P; on the other hand, thiourea, ineffective alone, markedly potentiated the *HO* effect in bone marrow. The DNA/RNA ratio remained unchanged in experiments with small amounts of *HO*, or in those with urethane or thiourea alone; it fell, however, in all those instances where the combined action of these substances caused

toxic symptoms in the animals and damage in the bone marrow, thus indicating that the DNA fraction suffered more under these conditions than RNA.

Effect of röntgen radiation on the phosphorus content in the bone marrow of rats and rabbits

In rats which received a single dose of radiation to the body as a whole, the nucleic acid P content of the bone marrow, and also of the spleen and testes, was examined at various times after exposure. The various aspects of these experiments are set out in Table 9 which illustrates the changes in total nucleic acid P from the 6th hour onwards after irradiation; Table 10 which provides data, based on a similar experimental series but with rather younger animals, concerning the DNA and RNA contents; Table 11 which summarizes the details of the general effects of irradiation as well as the chemical changes during a 10-week experimental period; and Fig. 1 which shows the DNA and RNA distribution in the bone marrow of rats irradiated with a single 500 r. dose of X-rays.

From the data in Table 9 it is evident that a few hours after irradiation there were gross changes in the appearance and consistency of the bone marrow owing to tissue damage which was accompanied by a fall in nucleic acid P, in some instances to about half the control value. The decline in nucleic acid content was most marked at 3–4 days, but low values persisted in most of the surviving animals for 2–3 weeks; thereafter, parallel with the gradual improvement in the general condition, and together with evidence of cellular recovery based on microscopic examination, there was a rise in the content of nucleic acid P, so that when examined at 50 days several, though not all, of the animals showed normal values for this phosphorus fraction (Tables 9

Table 9. *Effect of total body exposure to X-rays (500 r.) on total nucleic acid phosphorus in bone marrow, spleen and testes of rats*

(Averages based on values with three rats (range in brackets), except the 7-day value which is based on one rat only. Rats 200–225 g. at start of experiment.)

Time after exposure	Bone marrow (mg. P/100 g.)	Spleen		Testes	
		(mg. P/100 g.)	(mg. P/organ)	(mg. P/100 g.)	(mg. P/organ)
Untreated	264 (252–270)	179 (168–197)	1.7	66 (63–70)	0.65
6 hr.	133* (100–163)	—	—	—	—
24 hr.	123* (89–140)	—	—	—	—
4 days	78 (48–118)	82 (79–85)	0.45	71 (55–88)	0.9
7 days	82	130	0.62	79	0.8
21 days	158 (114–202)	275 (213–300)	3.6	51 (48–76)	0.5
50 days	250 (158–263)	177 (172–183)	1.2	46 (40–53)	0.65

* Bone marrow semi-fluid; admixture of blood.

and 10). The spleen underwent considerable fluctuations in weight as a result of irradiation; it was therefore of interest to present not only the concentration but also the content per organ of nucleic acid P; it was found that an early decrease was followed by a rise 3 weeks after irradiation which, however, was transient, so that more normal values were found at the later experimental stage (Table 9). In testicular tissue there were no marked early changes either in organ weight or in nucleic acid P level; at 3 weeks, however, a certain diminution in both became evident, which appeared progressive; this may well be linked with the fact that when spermatozoa were examined in irradiated surviving rats at 8–10 weeks after exposure they were almost completely immotile and largely disintegrated.

It will be noted that the DNA fraction appeared more sensitive to the damaging effect of radiation than the RNA (Table 11; Fig. 1). This applied not only to the bone marrow but also to the spleen, but was not very obvious in testicular tissue. Similar behaviour of the two nucleic acids was also observed in systemic *H* poisoning.

Fig. 1. Effect of total body exposure to a single dose of X-rays (500 r.) on the content of deoxyribonucleic acid (DNA) P (unshaded areas), and ribonucleic acid (RNA) P (shaded areas) in rat bone marrow.

Table 10. *Deoxyribonucleic (DNA) and ribonucleic acid (RNA) phosphorus content of rat bone marrow, spleen and testes, at various periods after whole body exposure to X-rays (500 r.)*

(Average values from two animals; some of the controls were examined at the beginning, the rest at the end of the experimental period. Rats, 150–200 g. body weight at the start of the experiment.)

Time after irradiation (days)	Bone marrow (mg. P/100 g.)		DNA RNA	Spleen (mg. P/100 g.)		DNA RNA	Testes (mg. P/100 g.)		DNA RNA
	DNA	RNA		DNA	RNA		DNA	RNA	
Untreated*	157	81	1.94	100	66	1.51	23	41	0.56
4	15	23	0.65	37	42	0.88	20	35	0.57
14	58	56	1.04	98	83	1.18	28	45	0.62
21	79	71	1.12	106	87	1.22	18	33	0.54
48	169	91	1.86	99	60	1.65	20	26	0.77
Untreated†	165	79	2.07	94	54	1.74	27	43	0.63

* At beginning of experiment.

† At end of experiment.

Table 11. *Late effects of body exposure to X-rays (500 r.) on the nucleic acid phosphorus content of rat bone marrow*

(Rats weighing 170–190 g. at start of experiment.)

Time after irradiation (weeks)	General condition	Total nucleic acid		DNA		RNA		DNA RNA
		(mg. P/100 g.)	(% loss)	(mg. P/100 g.)	(% loss)	(mg. P/100 g.)	(% loss)	
Untreated		267	—	179	—	88	—	2.0
1	Moribund; widespread haemorrhages	82	69	25	86	57	35	0.43
	Fair; slow gain of weight	215	19	130	27	85	4	1.53
2	Poor; loss of weight, offensive diarrhoea	128	52	64	64	64	27	1.00
	Poor; baldness, loss of weight	203	24	127	29	76	14	1.67
5	Fair; greyness, baldness, steady gain in weight	226	15	150	16	76	14	1.97
	Fair; greyness, sperms immotile	249	7	160	11	89	0	1.79
10	Fair; sperms immotile, growth of pigmented hair in bald areas	237	11	153	14	84	5	1.82

In several of these rats the liver content of glutathione and ascorbic acid as well as of glycogen was estimated during the early post-irradiation period, but no clear-cut results have so far been obtained. Livers of animals which succumbed to X-ray action with haemorrhagic manifestations contained no glycogen at all, but this may well have been due to poor food consumption.

Although rabbits were on the whole rather unsatisfactory experimental animals owing to the variability of their bone marrow P content, several experiments were carried out with litter mate rabbits belonging to the same breed. In Table 12 findings

DISCUSSION

Few data are available concerning the phosphorus content of bone marrow. The extensive and careful investigations of Davidson *et al.* (1947, 1948, 1950) deal with the content of nucleic acids in human bone marrow, obtained from normal individuals as well as from persons suffering from diverse forms of blood dyscrasias. It is not easy to compare results concerning bone marrow from the long bones of animals such as rats and rabbits with analyses based on human material aspirated from the sternum. Samples withdrawn by sternal puncture are likely to

Table 12. *Effect of body exposure to X-rays on the content of ATP and nucleic acid phosphorus in rabbit bone marrow*

(Two rabbit litters, A and B, of the same breed; irradiated with 1000 r.)

Litter	Time after irradiation (days)	Gross and microscopic appearance of bone marrow	ATP (mg. $\text{NH}_3\text{-N}/100 \text{ g.}$)	Nucleic acid (mg. P/100 g.)
A	Untreated	Normal, very cellular	1.73	89
	2	Slight, admixture of blood; mitotic abnormalities	1.12	51
	4	Gelatinous and oedematous; severe cell damage	0.52	12
	10	Oedematous and pale; signs of cellular recovery	0.82	23
B	12	Pale and 'fatty'; signs of cellular recovery	0.68	42
	16		0.70	54

Table 13. *Distribution of phosphorus compounds in rabbit bone marrow 3 hr. after total body exposure to X-rays (1500 r.)*

Litter mates	ATP amino N (mg./100 g.)	Acid-soluble P (mg./100 g.)		Nucleic acid P (mg./100 g.)	
		($\text{P}_{10} - \text{P}_0$)	$\text{P}_{\text{tot.}}$	DNA	RNA
Irradiated	0.18	3.0	28.6	27	15
Untreated	0.66	4.4	29.6	48	18

are recorded dealing with the behaviour of ATP and nucleic acid P after total body exposure to 1000 r. The animals showed few signs of distress in response to this dose, but there was a distinct decrease in the content of both these phosphorus compounds in the bone marrow, especially between the 2nd and 4th day; in this particular experiment even at 10 days values continued low as compared with those of the untreated litter mate, but in several other instances a nearly normal content of phosphorus was found at that stage. So far, experiments with rabbits have not been extended beyond 20 days; at that time there was a clear indication in some animals of complete, in others of partial, recovery of the bone marrow, both from the chemical and cytological point of view. A few experiments were carried out in which rabbits received larger doses of radiation (1500 r.) and the bone marrow was examined a few hours later, when the animals showed general symptoms of distress, some having intestinal disturbances; one such experiment is recorded in Table 13; ATP and nucleic acid P were already affected 3 hr. after irradiation, whereas total acid-soluble P remained unchanged.

contain a certain unavoidable and variable proportion of blood, whereas material from well bled animals usually has a negligible admixture of blood. Apart from technical details, anatomically distinct bones are known to contain marrow of different cellular compositions. But the greatest obstacle to comparison arises from the species differences in the make-up of the blood-forming tissue, such as were brought out by this investigation. The total nucleic acid P content of normal human marrow is given as 20.7 mg./100 g.; this is several times less than in rabbit, and about ten times less than in rat bone marrow. The DNA/RNA ratio in the bone marrow of normal rats varied within relatively narrow limits from 1.6 to 2.0; human material appears to show a wider range, 0.5–2.3, the average (1.3) being below that found in the animal species so far investigated.

Some determinations of the phosphorus content of rabbit bone marrow have also been described by Dietz (1949). However, his technique of phosphorus estimation was such as to render impossible interpretation in terms of the phosphorus fractions accepted for the experiments presented here. In the paper referred to the phosphate was determined as

follows: the bone marrow sample was first ground for an unspecified length of time in a mortar, then mixed with 5 vol. of water, and allowed to stand at room temperature for 1 hr.; after this trichloroacetic acid was added and in the protein-free filtrate 'phosphate' was precipitated as strychnine phosphomolybdate and determined by means of the colorimetric method used by Tisdall (1922) for phosphate estimations in serum. However, in serum total acid-soluble and directly determinable P are nearly identical, whereas in the bone marrow, in addition to small amounts of inorganic phosphate, there are several organic acid-soluble phosphorus derivatives, the content of which can only be assessed after incineration. Quite apart from that, 1 hr. incubation period between the collection of the tissue and the phosphate assay leads to changes in the pattern of phosphorus distribution owing to the presence in bone marrow of enzymes which act upon various phosphorus compounds of the tissue. There is, therefore, no common ground for the comparison of phosphorus in the protein-free filtrate from rabbit bone marrow. However, Dietz's values for 'total P' (obtained after incineration of whole tissue) do in some instances agree with those reported in this study. It would seem, however, that on the whole the assay of a more strictly defined phosphorus fraction, such as for instance nucleic acid P, is preferable as a basis for comparative studies in large numbers of animals to determinations of the 'total P', which, being made up of acid-soluble phospholipin, phosphoprotein and nucleic acid P, can be expected to vary widely.

The usefulness of the nucleic acid P value as an indicator of the metabolic potential of bone marrow was illustrated in experiments with myelotoxic agents such as mustard gas and X-rays, as well as in those which showed the influence upon the bone marrow of qualitative and quantitative changes in the food composition. The severe initial clinical symptoms caused by the toxic agents were accompanied by a decrease in the content of nucleic acid P in the bone marrow; as reported earlier (Lutwak-Mann & Gunz, 1949), this coincided with markedly lowered respiration and glycolysis of the bone marrow; in the surviving animals, parallel with improvement in the general condition, a gradual return to near normal values was observed in nucleic acid P; manometric measurements also indicated recovery in the respiratory and glycolytic functions of the bone marrow. Separation of nucleic acid into DNA and RNA made it possible to assess the relative sensitivity of these two fractions towards the damaging factors; judging from the DNA/RNA ratio it would seem that as a result of whole body exposure to X-rays, or in systemic mustard gas poisoning, DNA suffered more than RNA; the ratio fell sharply in the early experimental stage, but gradually

returned to nearly normal values in animals which withstood the damage. It is, however, conceivable that other experimental conditions, for example, multiple small doses of radiation, or radiation applied more specifically to bone marrow-carrying areas and not to the body as a whole, would provoke in the blood-forming tissue a reaction which would result in a different pattern of distribution of these phosphorus compounds. The possibility must also be envisaged of pathological changes in the bone marrow which would find their expression, e.g. in reduced ability to form pigment without, perhaps, much change in cellularity and nucleic acid concentration.

Although nutritional deficiency considerably lowered the level of total nucleic acid P in the bone marrow, it has not yet been possible to establish any accompanying characteristic change in the DNA/RNA ratio. It is highly probable that deficiencies other than lack of vitamins of the B group would also lead to changes in the bone marrow; wider studies will perhaps bring out more clearly which of the two nucleic acids is more affected by dietary insufficiency.

During autolysis of bone marrow *in vitro*, RNA appeared to be more susceptible than DNA to enzymic attack, with the result that the DNA/RNA ratio became nearly double in the course of a few hours' incubation at 38°. The source of the ammonia which appeared at the same time has not yet been established; while it is certain that only a small proportion of this ammonia could have been derived from preformed ATP, it is not unlikely that some at any rate was connected with the breakdown of nucleic acids, more particularly of RNA. The processes observed during *in vitro* incubation of bone marrow were not limited to this tissue, as they were found to occur in spleen, though to a lesser extent.

The pharmacodynamically interesting experiments showing the potentiation of the effect of small, *per se* ineffectual doses of HO by DMT and DET, thiourea and urethane, lack for the time being adequate explanation of the mechanism involved, though the formation *in vivo* of H or a substance with H-like properties is very probable. In an attempt to approach this problem, several (unpublished) *in vitro* experiments were set up to see whether mixtures of HO with the thiocarbamates would inhibit bone marrow glycolysis in a manner similar to H; also mixtures of varying amounts of HO and DMT were first incubated with liver and kidney slices, and then bone marrow glycolysis was measured in these incubation fluids; however, it was not possible to reproduce *in vitro* conditions resembling those in the whole animal. Moreover, as reported above, the breakdown of bone marrow nucleic acids *in vitro* was not influenced by H, HO, or HO + DMT. In this connexion it is of interest to recall the work of

Peters & Wakelin (1947), who found that the *in vitro* inhibitory effect of mustard gas, and other vesicants with active chlorine atoms, towards the pyruvic oxidase system could be substantially increased by DET. This was found to be due to the formation of 2-chloroethyl-2-(*N*-diethyldithiocarbamate)ethyl sulphide, which was isolated in crystalline form, but which, however, had no toxicity in rats or guinea pigs.

From the practical point of view it is worthy of note that urethane (to which rats have been found to be remarkably resistant even on prolonged administration) caused a decline in nucleic acid P in the bone marrow, if combined with small, non-toxic amounts of *HO*; to establish whether the application of urethane 'fortified' with *HO* is suitable for clinical purposes, e.g. in the treatment of leukaemia, would necessitate extensive further laboratory as well as clinical trials.

SUMMARY

1. A study has been made of the phosphorus content in the bone marrow of rabbits and rats, with special reference to acid-soluble, adenosinetriphosphate, and nucleic acid phosphorus. The effect of certain experimental conditions upon the distribution of the various phosphorus fractions has been investigated, both in the whole animal and *in vitro*.

2. During *in vitro* incubation, enzymes present in the bone marrow caused several changes in the distribution of acid-soluble and nucleic acid phosphorus. There was a decrease in difficulty hydrolysable phosphorus esters accompanied by an accumulation of inorganic phosphorus; an increase in total acid-soluble phosphorus, particularly marked in rat bone marrow, and a fall in nucleic acid phosphorus,

chiefly at the expense of ribonucleic acid; adenosinetriphosphate was relatively stable under aerobic conditions, but broke down more rapidly in absence of oxygen. Considerable amounts of ammonia, which exceeded many times the content of preformed adenosinetriphosphate amino nitrogen, were formed, especially in rat bone marrow.

3. Alterations in food composition leading to undernourishment or vitamin deficiency in rats markedly lowered the content of nucleic acid phosphorus in the bone marrow.

4. The behaviour of phosphorus compounds in the bone marrow was investigated at various stages of systemic poisoning with mustard gas and mustard sulphoxide, and also in rats and rabbits which received single doses of X-rays to the whole body. As a result of exposure of animals to these agents, there was a pronounced fall in nucleic acid phosphorus in the bone marrow; changes in the deoxy-ribonucleic/ribonucleic acid ratio suggested that deoxyribonucleic acid was more strongly affected than ribonucleic acid; a lowered content of adenosinetriphosphate and total acid-soluble phosphorus was also observed. In surviving animals, parallel with general recovery, there was a gradual return to normal in these phosphorus fractions of the bone marrow.

5. The significance of nucleic acid phosphorus assay as an indicator of the metabolic potential of the bone marrow is discussed.

This investigation was supported by a personal and material expenses grant from the Medical Research Council. The co-operation of Miss R. Leader in the animal experiments is acknowledged with gratitude. The author's thanks are due to Dr E. Kodicek, Dr F. W. Gunz and Dr R. C. Campbell, for their help in the nutritional, histological and statistical aspects of the study, respectively.

REFERENCES

- Cameron, G. R. & Rydon, H. N. (1944). Porton Report.
 Davidson, J. N., Leslie, I. & White, J. C. (1947). *Biochem. J.* **41**, xxvi.
 Davidson, J. N., Leslie, I. & White, J. C. (1948). *J. Path. Bact.* **50**, 1.
 Davidson, J. N., Leslie, I. & White, J. C. (1950). *Biochem. J.* **47**, xvi.
 Dietz, A. A. (1949). *Arch. Biochem.* **23**, 211.
 Dietz, A. A. & Steinberg, B. (1949). *Arch. Biochem.* **23**, 222.
 Evans, J. D. & Bird, R. M. (1949). *J. biol. Chem.* **181**, 357.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Goldinger, J. M., Lipton, M. A. & Guzman Barron, E. S. (1947). *J. biol. Chem.* **171**, 801.
 Kodicek, E. & Carpenter, K. J. (1950). *Blood*, **5**, 522.
 Lutwak-Mann, C. (1947*a*). *Biochem. J.* **41**, xxx.
 Lutwak-Mann, C. (1947*b*). Report to the Ministry of Supply by Dr Malcolm Dixon, no. 32.
 Lutwak-Mann, C. (1949). *Nature, Lond.*, **164**, 607.
 Lutwak-Mann, C. (1951). *Biochem. J.* **48**, xxv.
 Lutwak-Mann, C. & Gunz, F. W. (1949). *Biochem. J.* **44**, iii.
 Lutwak-Mann, C. & Mann, T. (1950). *Nature, Lond.*, **165**, 556.
 Lutwak-Mann, C. & Mann, T. (1951). *Biochem. J.* **48**, xxvi.
 Needham, D. M., Cohen, J. A. & Barrett, A. M. (1947). *Biochem. J.* **41**, 631.
 Parnas, J. K. & Lutwak-Mann, C. (1935). *Biochem. Z.* **278**, 11.
 Peters, A. R. & Wakelin, R. W. (1947). *Biochem. J.* **41**, 545.
 Schmidt, G. & Thannhauser, S. J. (1946). *J. biol. Chem.* **161**, 83.
 Schultze, M. O. (1941). *J. biol. Chem.* **138**, 219.
 Tisdall, F. F. (1922). *J. biol. Chem.* **50**, 329.
 Warren, C. O. (1934). *Amer. J. Physiol.* **128**, 455.
 Warren, C. O. (1940). *Amer. J. Physiol.* **131**, 176.