Of practical importance is the fact that no loss of aneurin from the scutellum as a result of soaking wheat in water could be detected. Doubt has existed whether the moistening of wheat grain, carried out to adjust suitably the moisture content in preparation for milling, might not produce a redistribution of the aneurin so that the endosperm might gain in the vitamin from the scutellum, which would consequently be of less importance. Such does not seem to be the case; the scutellum will be as important in moistened wheat as in dry. It follows likewise that adjustment of water content as a preparation for milling can be undertaken freely without affecting the validity of any technical development based on the high vitamin content of the scutellum.

A further discussion of the practical aspect of the subject, which should be read in the light of the extended range of figures given in the present paper, has been given by Moran (1942).

There is as yet no indication of the metabolic significance of this concentration of aneurin; no clear conclusions can be drawn from the figures obtained on germinating wheat. There was very little change in aneurin content of the scutellum at 3 days' germination, at which time the dry weight of the embryo had increased three times and erosion of the endosperm was well marked. At 5 days' germination, at which time the aneurin had fallen by half, the dry weight of the embryo had increased nine times and erosion of the endosperm was well advanced. At this time, the scutellum showed signs of physical change, being dark coloured, horny and brittle, though there was little change in dry weight.

SUMMARY

1. The aneurin content of the scutellum and embryo portion of the germ of various wheat types and of rye, barley, oats and maize has been determined.

2. Expressed as an average for all the wheats, the scutellum was 1·5 % of the grain weight and contained 59 % of the total aneurin in the grain, whilst the embryo was 1·2 % of the grain weight and contained 3 % of the total vitamin. Corresponding figures for the other cereals were of the same order.

3. No loss of aneurin from the scutellum could be detected in wheat which had been soaked in water for up to 12 hr.

4. Analytical figures relating to total N, total P, phytate P, phosphatase and petrol ether extract for scutellum and embryo of wheat are given. The scutellum contained 1·3 % phytate P, which was more than three times that of the embryo, and 30 % lipoid material, which was double that of the embryo.

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The Metabolism of Azo Compounds

1. AZOBENZENE

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(*Received* 7 *April* 1944)

B. Fischer (1906) described epithelial proliferation following the injection of the azo dyestuff scarlet red. Although the growths were non-malignant he rightly claimed his discovery as the first case of the induction of tumour-like proliferation by a chemical compound. Hayward (1909) found that the active part of the scarlet red molecule was represented by 4′-amino-2:3′-azotoluene, and Yoshida (1934) produced proliferation of liver tissue in mice and liver tumours in rats with this substance. Kinosita (1937) later examined other derivatives of azobenzene and found *p*-dimethylamino-azobenzene to be highly active in producing liver tumours; and bladder tumours were produced with 2:3′-azotoluene.

In other attempts to produce bladder cancer, Cook, Hewett, Kennaway & Kennaway (1940) selected the three azonaphthalenes, on the supposition that these might arise by oxidation of naphthylamines and so be a factor in the occupational incidence of the disease in chemical workers. No bladder tumours were obtained, but liver changes in mice, similar to those induced by the azobenzene derivatives, resulted from treatment with 2:2′-azonaphthalene. To explain the carcinogenic action of this compound they suggested that it might be reduced in the body to 2:2′-hydrazonaphthalene, which, by a 'benzidine' type of rearrangement is converted to 2:2′-diamino-1:1′-dinaphthyl, and subsequently to 3:4:5:6-dibenzcarbazole, which may be the real carcinogenic agent. This suggestion is supported by the fact that 2:2′-diamino-1:1′-dinaphthyl was itself found to be capable of inducing liver tumours in mice and 3:4:5:6-dibenzcarbazole had been shown to produce skin cancer and malignant connective tissue tumours as well as liver tumours (Boyland & Brues, 1937; Andervont & Edwards, 1941). It thus provides a remarkable linkage between carcinogenic substances of the azo class, and the polycyclic compounds.

No direct evidence that such metabolic changes can take place in the body has, however, hitherto been produced, and we therefore began a systematic study of the metabolism of azo compounds, starting with azobenzene, the simplest member of the group.

METHODS

Administration to rats. Twenty adult male rats were given 50 mg. azobenzene, dissolved in 0·5 ml. arachis oil, by intraperitoneal injection. As this appeared to be well tolerated the dose was increased to 75 mg. for subsequent injections (100 mg. azobenzene had been found previously to be toxic, causing death of a number of the animals). The injections were repeated every 3 or 4 days, the animals being kept in metabolism cages and the urine collected.

Colorimetric estimation of amines. Amino metabolites were estimated in the urine by a colorimetric method based on the colours obtained by coupling the diazotized amines with *N*-sulphatoethyl-*m*-toluidine, a reagent which has been found by Rose & Bevan (1944) to have advantages over the usual coupling components in sulphanilamide estimations. The colours obtained were compared by means of a Hilger 'Spekker' Photoelectric Absorptiometer, and by the use of Ilford 'Spectrum' filters rough absorption curves were obtained.

Reagents. (1) $NaNO_2$, 0·1%. (2) Sodium acetate, $C_2H_3O_2Na.3H_2O$, 20%. (3) *N*-sulphatoethyl-*m*-toluidine, 1%. All as aqueous solutions.

Method of estimation. 1 ml. of the urine is treated with 0·1 ml. of conc. HCl followed by 1 ml. of 0·1% $NaNO_2$. After 2 min., 2 ml. of 20% sodium acetate are added, 1 ml. of the *N*-sulphatoethyl-*m*-toluidine reagent, and 5 ml. of ethanol. After being mixed and allowed to stand for 10 min. for development of the colour (usually yellow at this stage), the solution is acidified by addition of 0·5 ml. conc. HCl, giving the red colour which is estimated on the colorimeter. For determination of colour in the urine after hydrolysis, 6 ml. of the urine were treated with 1 ml. conc. HCl and hydrolyzed by gentle boiling for 5 min. The precipitate which formed was removed by centrifuging and the colour estimation carried out as described on 1 ml. of the clear supernatant liquid.

RESULTS

The urine from the azobenzene-treated rats, when diazotized and coupled with *N*-sulphatoethyl-*m*-toluidine, gave an intense red purple colour from which a purple precipitate soon separated out. Neither aniline nor *p*-aminophenol gave this purple precipitate, and moreover the purple colour was found to dye cotton, for which aniline type azo dyes have no affinity. Benzidine was found to give a coloured substance with the coupling reagent, which was identical in properties with that obtained from the urine.

Extraction of the urine. The urine (750 ml.), which was alkaline to litmus, was extracted with ether in a continuous extractor for 40 hr. The ether extract, after evaporation of the ether, was treated with HCl (1 ml. of conc. HCl diluted with 5 ml. water). Some oil remained undissolved and this was separated by filtration, with the aid of a little charcoal. After washing with water the volume of the filtrate was 20 ml. 0·1 ml. of this solution subjected to the colour test gave a red colour for which a colorimeter reading E_{green} 1·5 was obtained. On standing, a small amount of purple precipitate separated out. The remainder of the solution was made alkaline with NaOH and steam distilled. The distillate gave a bright red colour reaction, 0·15 ml. of the first runnings giving the colorimeter readings shown in Fig. 1, which includes for comparison the colour

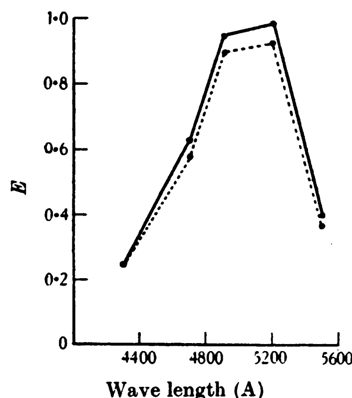


Fig. 1. Absorption curves obtained by the use of the 'Spekker' photoelectric absorptiometer on the colours obtained from the steam distillate of the ether extract of the urine of rats treated with azobenzene, and from aniline. —— Steam distillate from ether extract of azobenzene urine. ----- Aniline (0·025 mg.)

readings obtained with aniline, when diazotized and coupled with *N*-sulphatoethyl-*m*-toluidine under identical conditions. There is very close agreement, the curves for aniline and the metabolic product being practically identical.

Steam distillation was continued until the distillate gave only a faint colour reaction. 0·25 ml. of the total distillate (330 ml.) gave a colorimeter reading E_{green} 0·85, from which it was estimated that the total amount of aniline present was about 33 mg.

The residual alkaline urine after this first ether extraction was acidified with conc. HCl (125 ml.) and heated on the steam bath for 1 hr. After removal by centrifuging of the precipitate which formed, the clear liquid was made alkaline with NaOH (50 g. dissolved in a little water) and again extracted with ether in the continuous extractor for 40 hr. The ether extract, after removal of the ether, was boiled with a little water with addition of a small amount of charcoal and filtered. On cooling, crystals separated from the filtrate. These were filtered off and recrystallized from water. After drying at 110° the m.p. was 123–125°; mixed m.p. with benzidine (m.p. 127°) gave m.p. 124–126°.

Sulphur and glucuronic acid estimations

Quantitative estimations of the daily inorganic sulphate, ethereal sulphate, neutral sulphur and of the glucuronic acid excretion were carried out. For the glucuronic acid estimations the method of Maughan, Evelyn & Browne (1938) was used and the sulphur estimations were carried out by a modification of the usual benzidine method, details of which will be published later.

Ten young male rats were kept on a standard diet for a week before treatment with azobenzene, urine being collected daily and S and glucuronic acid estimations being carried out. The animals then each received 50 mg. azobenzene by intraperitoneal injection and the daily estimations of S and glucuronic acid were continued. The results are given in Table 1.

Table 1. *Excretion of* S *and of glucuronic acid by rats before and after administration of azobenzene*

Results are expressed as mg. of S or of glucuronic acid/100 g. rat/day.

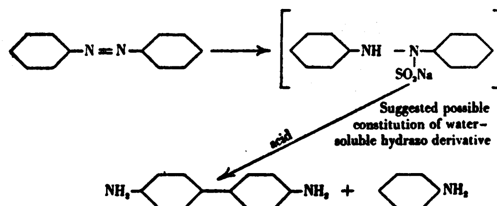
Days after injection	Inorganic S	Ethereal S	Neutral S	Glucuronic acid
Control period. Ten male rats each given intraperitoneally 1 ml. arachis oil				
1	1·4	0·18	0·48	7·5
2	2·9	0·28	0·78	9·4
3	3·0	0·34	0·64	13·0
Experimental period. The above ten rats each given intraperitoneally 50 mg. azobenzene in 1 ml. arachis oil				
1	1·3	0·32	1·7	14·5
2	0·1	1·40	1·8	31·4
3	3·8	0·95	4·3	35·4
4	6·6	0·27	2·5	25·0

The injection of azobenzene results in a marked rise in the ethereal sulphate and in the glucuronic acid excreted, maximum values being obtained on the second and third days after injection. The amount of amine excreted as measured by the colour reaction also attains a maximum during this period. A considerable increase in the excretion of neutral sulphur occurs, reaching a maximum on the third day. A similar increase in neutral sulphur excretion has been observed in rats treated with the carcinogenic *p*-dimethylamino-azobenzene and this is being further investigated.

DISCUSSION

From the urine of rats injected with azobenzene the products which have actually been isolated are aniline and benzidine. By ether extraction of the unacidified urine (which was slightly alkaline) only aniline was identified in the extract, the colour reaction indicating the presence of only a minute trace of benzidine. The benzidine which was subsequently isolated must therefore be derived from a water-soluble product, present in the original urine, but not extractable with ether. On acidification with dilute acid, benzidine is formed from this water-soluble product and, after the solution has been made alkaline, can readily be extracted with ether.

To obtain benzidine from azobenzene, reduction to hydrazobenzene must first take place, and to explain the above facts we suggest that this reduction takes place in the body, the hydrazobenzene being then rapidly excreted as a water-soluble derivative, formed possibly by conjugation with sulphuric acid as a N-SO_3Na derivative, or with glucuronic acid in a similar manner.



This derivative on treatment under fairly mild acid conditions is assumed to undergo the benzidine rearrangement. It is possible that this rearrangement can take place to some extent *in vivo*, particularly in certain organs such as the liver. In fact the liver of azobenzene-treated rats, after being heated with alkali and extracted with ether, yielded an extract which gave a faint colour reaction for

benzidine. The amount present in the liver must be very small, however, and it is evident that the main tendency is for azobenzene to be rapidly eliminated as the water-soluble hydrazo derivative.

Azobenzene is not carcinogenic, but in work which is being carried out with the carcinogenic *p*-dimethylamino-azobenzene evidence has been obtained that similar metabolic changes take place. In this case, however, the hydrazo derivative does not appear to be so stable and undergoes the benzidine rearrangement in the body more readily, since a substance giving the colour reaction of one of the 'benzidine change' products of *p*-dimethylamino-azobenzene can be extracted directly from the neutral or alkaline urine.

SUMMARY

The urine of rats which have received azobenzene has been found to contain aniline, and a water-soluble derivative which, after acidification with dilute acid followed by addition of sodium hydroxide and subsequent extraction with ether, yields benzidine.

We desire to thank I.C.I. (Dyestuffs) Ltd. for permission to publish this work. We are also indebted to the Sir Halley Stewart Trust for a Fellowship held by one of us (F. L. W.) during this work and to the British Empire Cancer Campaign and to the Anna Fuller Fund for generous grants which have helped to support this investigation.

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Changes in the Skeleton during Gestation and Lactation in the Rat

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(*Received* 3 *March* 1944)

Many observations show that during pregnancy mammals store Ca and P in the skeleton and that these substances are released during the ensuing lactation (Forbes, Black, Braman, Frear, Kahlenberg, McClure, Swift & Voris, 1935; Coons, Schiefelbusch, Marshall & Coons, 1935; Duckworth & Warnock, 1942–3; Goss & Schmidt, 1930). The extensive growth of cancellous bone which takes place during the pre-ovulatory stage with subsequent resorption about the time of ovulation in the pigeon suggests that a similar process occurs also in birds (Kyes & Potter, 1934; Bloom, Bloom & McLean, 1941). The classical studies of Bauer, Aub & Albright (1929) showed that cancellous bone served as a store of Ca and P and that storage and depletion were governed by the level of Ca intake. Using alizarin red as an intravital stain, they also demonstrated that mobilization of the trabeculae was caused by parathyroid hormone.

Apparently, no investigation has been specifically designed to determine if the trabeculae are the sole physiological store of Ca and P in the adequately nourished animal, or whether, in lactation, the shaft is also drawn upon by direct resorption, or by partial demineralization without resorption, or by resorption with concomitant partial demineralization of the unresorbed portion. The importance of this fundamental question is underlined by the description given by Theiler & Green (1931–2) of the modern, high milk-producing, dairy cow: 'such an animal is pathological, oscillating between a compact bone tissue and a mildly osteoporotic framework in each reproductive cycle.' In addition no comparison seems to have been made of the behaviour of the skeleton of animals lactating normally with that of animals which, at the conclusion of gestation, are not allowed to suckle their young. Neither have comparative studies been made of the rates of replenishment of skeletal stores after lactation in pregnant and non-pregnant animals.

The present investigation was designed to examine these points. The shafts of certain long bones were separated from the ends and the two portions studied. Although this mode of approach has obvious limitations, it permitted some clarification of the problem.