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Studies in Detoxication

32. THE INFLUENCE OF BROMOBENZENE AND CYSTINE ON THE BROMINE CONTENT OF THE HAIR OF RATS

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The ultimate objective of this investigation was to find out how orally administered compounds possessing therapeutic activity could be made to appear in the skin and hair. It was thought that some evidence concerning this could be obtained by feeding compounds which form mercapturic acids *in vivo*, because cystine is much involved in the elaboration of hair and skin and in the formation of mercapturic acids. For this study, bromobenzene was selected as the mercapturic acid former, because it could be traced by its bromine content. It was possible that *p*-bromophenylmercapturic acid might find its way to the skin and hair in quantities sufficient to warrant studying, in a similar manner, mercapturic acid formers carrying therapeutic groups.

It has long been known that arsenic, when administered to animals, appears in the hair, skin and nails, and this is explained by the fact that arsenic compounds combine with sulphydryl groups *in vivo*. Haddow, Elson, Roe, Rudall & Timmis (1945) have shown that when the flavine, 9-phenyl-5:6-benzo-*iso*alloxazine, is injected into albino rats, an orange-yellow pigmentation of the growing parts of the hair appears. The pigment of the hair was shown to be either the original flavine or a simple derivative spectroscopically indistinguishable from it. The results of analyses of the sulphur compounds (inorganic and ethereal sulphates and neutral sulphur) of the urine of the injected animals were consistent with mercapturic acid formation, and it was suggested that the flavine might have been transported to the hair in combination with the cystine used for the elaboration of keratin. Furthermore, Crabtree (1944, 1945, 1946) has shown that a number of mercapturic acid formers, including bromobenzene, when applied locally to the skin, are probably detoxicated by mercapturic acid formation in the skin. At the same time there is a temporary decrease in the glutathione content of the skin and a delay in chemical carcinogenesis caused by 3:4-benzpyrene applied simultaneously. There is therefore ample evidence of a close connexion between the metabolism of mercapturic acid formers and keratin containing tissues.

In the present work, the plan was to feed varying amounts of bromobenzene and L-cystine to different groups of rats which had been clipped free of hair at the beginning of the experiment, and then, after an interval sufficient to allow the new hair to grow to normal length, to analyse the hair and certain organs for bromine and the hair for cystine. It will be shown that in animals showing signs of cystine deficiency as a result of feeding bromobenzene, the bromine content of the hair is much higher than that of other tissues examined.

METHODS

Animals. Twenty-four female albino rats (average weight 110–120 g. and 3–3·5 months old) were divided into six groups, the total weight of rats in each group being approximately equal. Each rat was individually marked, and periodically weighed.

At the beginning of the experiment each rat was clipped (under ether anaesthesia) as closely as possible with electric clippers over the whole of the body excluding the head forward of the ears. After weighing, each group was placed in a wire cage over a large funnel which had been coated with paraffin wax for better drainage of urine. A glass ball, suspended just below the orifice of the funnel, served to separate the faeces from the urine.

On the 30th day after the beginning of the experiment three rats in each group were killed by a blow on the back of the head. The hair was clipped, and the liver, kidneys and muscles of the thigh were removed and stored in 95% ethanol overnight.

One rat in each group was kept for 44 days after the beginning of the experiment, but from the 30th day it was fed on normal stock diet with no added cystine or bromobenzene. On the 44th day it was killed and its hair and tissues analysed for bromine.

Diet. The stock diet consisted of Lever cubes. In our experience this diet is nutritionally sound and rats breed and grow well on it. On analysis these cubes were found to contain approximately 0·5% cystine, 6% fat, 17% protein, and 10% moisture. The bromine content was found to be 34 μg./g. The amount of food eaten each day was determined in a pilot experiment and the rats were thereafter given a definite amount of food each morning. The rats gradually increased their food consumption as they became accustomed to the bromobenzene. At first they ate 10 g. a day and lost weight; later they ate 14 g. a day. The food containers were so arranged that loss of food by spilling was negligible. Water was allowed *ad lib.*

Bromobenzene (b.p. 156°) and L-cystine were administered by mixing with the ground Lever cubes. Cystine was mixed with the food by stirring and shaking. The bromobenzene was at first added to the dry food from a microburette, but it was soon clear from the mercapturic acid output (Table 2) that incomplete mixing enabled the rats to pick out particles of food not contaminated with bromobenzene. Another method of mixing was tried and used. From the sixth day of the experiment, the bromobenzene was measured into a test tube, shaken up into an emulsion with water and then thoroughly mixed with wetted food powder. As a result there was an immediate rise in mercapturic acid output. The amounts of L-cystine and bromobenzene added to the diet of each group is shown in Table 1.

Table 1. *Amounts of* L-*cystine and bromobenzene added to diet of rats*

Group	Cystine added (g./100 g. of diet)	Bromobenzene added (g./100 g. of diet)	Bromobenzene (approximate dose; g./kg. of rat)
I	0·2	0·5	0·6
II	0·4	1·0	1·2
III	0·7	1·5	1·8
IV	1·0	2·0	2·4
V	0	1·5	1·8
VI	0	0	0

Determination of p-*bromophenylmercapturic acid in urine.* The method used was that of Stekol (1936) with minor modifications. The urine of each group of rats was collected each day just before feeding. The sides of each funnel were washed with 25 ml. of 0·05 N-NaOH and the washings added to the urine which was then stored in the refrigerator. Pooled 3-day urines were analysed.

For the determination of mercapturic acid, the urine of each group was centrifuged, and the dark brownish red clear urine was transferred to a 250 ml. measuring flask. The residue in the centrifuged tube was washed twice with 10 ml. of dilute NaOH, centrifuged and the washings added to the main bulk of urine which was diluted to 250 ml. with water.

Table 2. p-*Bromophenylmercapturic acid excretion by rats receiving bromobenzene with and without cystine*

(Dose of bromobenzene: group I, 0·6 g./kg.; groups III and V, 1·8 g./kg. Groups I and III received cystine, group V did not. Figures for groups II and IV are not included in this table (but see Fig. 2). Group VI (controls) excreted no mercapturic acid. Each group contained 4 rats. M.A. = *p*-bromophenylmercapturic acid.)

	Total M.A. excreted in 3 days (g.)			M.A./rat/day (mg.)			Bromobenzene conjugated as M.A. (% of dose)		
Group ...	I	III	V	I	III	V	I	III	V
Period (days)									
1–3	0·115	0·331	0·288	9·6	27·6	24	9·6	9·2	8·0
4–6	0·172	0·403	0·360	14·4	33·6	30	14·4	11·2	10·0
7–9	0·423	1·004	0·879	36	84	73	25·2	19·6	17·2
10–12	0·454	1·307	0·734	38	109	61	26·4	25·6	14·4
13–15	0·505	1·437	0·615	42	120	51	29·2	28·0	12·0
16–18	0·553	1·562	0·611	46	130	51	32·4	30·4	—
19–21	0·564	1·701	0·517	47	142	43	33·2	33·2	—
22–24	0·600	1·639	0·378*	50	137	42	34·8	32·0	—
25–28	0·576	1·599	0·351†	48	133	39	34·0	31·2	—
28–30	0·562	1·557	0·222†	47	130	37	33·8	30·4	—
Total	4·524	12·540	4·755	—	—	—	—	—	—

* Three rats. † Two rats.

Table 3. *Bromine content of the tissues of rats receiving bromobenzene in the diet*

(The stock diet of Lever cubes contained 34 μg. of Br/g.)

	Bromine (μg./g. of tissue)					
	Hair		Muscle		Liver	
Rats killed on ...	30th day	44th day	30th day	44th day	30th day	44th day
Group						
I	149	165	135	71	106	58
II	178	206	122	59	165	69
III	174	214	147	74	151	71
IV	236	295	173	93	168	78
V	605	702	112	54	101	64
VI (controls)	49	47	28	27	35	37

A 50 ml. sample was now treated (to remove interfering substances) with 25 ml. of 20% (w/v) $ZnSO_4$, the mixture vigorously shaken, and 25 ml. of 0·5N-NaOH added with shaking. The precipitate was removed by filtration, leaving a pale straw-coloured filtrate. For the blank titration 10 ml. of the filtrate, 10 ml. of distilled water, 10 ml. of 95% ethanol, 4 ml. of 2·5N-HCl and 10 drops of starch solution were mixed and cooled in an ice-salt bath. The solution was titrated with 0·1N-iodine until a pale blue colour persisted for 30 sec.

For the test titration 10 ml. of the filtrate and 4 ml. of 2·5N-NaOH was heated on a sand bath for 30 min. The mixture was cooled and finally placed in an ice-salt bath. Water (10 ml.), ethanol (10 ml.), 2·5N-HCl (10 ml.) and 10 drops of starch were added and the mixture was titrated with 0·01N-iodine while still in the ice-salt bath. The weight of mercapturic acid in a 3-day urine (in mg.) = (test titre − blank titre) × 3·18 × 50 (318 being the molecular weight of *p*-bromophenylmercapturic acid). The reagents were standardized and recoveries from urine checked against pure *p*-bromophenylmercapturic acid (m.p. 152°) prepared from rat urine according to McGuinn & Sherwin (1933). Recoveries of this acid added to water, normal rat urine and the urine of rats receiving bromobenzene ranged from 96 to 102%.

The mercapturic acid outputs for the five groups are given in Fig. 2 and a complete set of figures for groups I, III and V is given in Table 2.

Distribution of bromine

Hair. For analysis, the hair was well washed with water, then extracted with 95% ethanol for 4 hr. followed by ether for 4 hr. and finally dried *in vacuo* and weighed. The weights of the hair at the beginning and end of the experiment are given in Table 4.

Liver and muscles. These tissues, after keeping overnight in 95% ethanol, were homogenized with acetone in the Waring blender, centrifuged and the acetone layer poured off. The residue was dried at 110° for 6 hr. and kept in stoppered bottles until analysed.

Analysis. Bromine in the hair and tissues was determined by the method of Winneck & Smith (1937*a*) with slight modification. This method entails alkaline ashing of the materials followed by conversion of bromide to bromate which is estimated by iodine-thiosulphate titration. Between 0·1 and 1·0 g. of material was ashed with 1 ml. each of 5N-KOH, 5N-K_2CO_3 and 20% sucrose solution. To ensure complete ashing a temperature of 600° was used. The results are given in Table 3.

Cystine content of the hair

The hair was analysed for cystine by hydrolysing for 8 hr. with HCl and formic acid (Miller & du Vigneaud, 1939) and subsequently determining the cystine in the hydrolysate by the method of Folin & Marenzi (1929).

The hair of group V (Table 5) contained 8% less cystine (i.e. 12 instead of 13%) than other groups, and from the work of Lightbody & Lewis (1929) these animals therefore exhibit a cystine insufficiency. Under the microscope, the hair of group V showed a smaller proportion of cortex than the controls, further evidence of cystine deficiency (Smuts, Mitchell & Hamilton, 1932).

Table 4. *Weight of cleaned and dried hair of rats obtained at the beginning and end of the experiment*

(Values for the beginning of the experiment and on the thirtieth day are means for four and three rats respectively; values on the forty-fourth day are for one rat only.)

	Weight of hair/rat (g.)		
Group	At beginning	On 30th day	On 44th day
I	1·963	1·517	1·75
II	1·839	1·603	1·87
III	2·087	1·399	2·13
IV	2·366	1·659	2·01
V	1·909	1·327	1·91
VI	2·256	1·724	2·29
Average	**2·07**	**1·54**	**1·99**

Table 5. *Cystine content of the hair of rats receiving varying amounts of* L-*cystine and bromobenzene*

	Percentage cystine in hair of rats killed on	
Group	30th day	44th day
I (cystine + PhBr)	13·13	13·16
II (cystine + PhBr)	13·18	13·12
III (cystine + PhBr)	13·25	13·29
IV (cystine + PhBr)	13·28	13·24
V (PhBr only)	12·29	12·02
VI (controls)	13·16	13·20

As regards the weight of the hair, it is clear from Table 4 that none of the groups had recovered their full weight of hair in 30 days although the recovery was about 75%. However, if the results for the single remaining rat in each

group are acceptable, then the full weight of the hair has been recovered sometime between the thirtieth and forty-fourth day. The low figure for group V on the thirtieth day is due to the fact that it is made up of hair from three rats, that lived for different periods, i.e. 21, 27 and 30 days, and is probably of no significance.

RESULTS

The growth curves of the rats during the first 30 days of the experiment are shown in Fig. 1. All groups show a loss of weight during the first 12 days, although the loss in the control group VI was slight and ceased after 6 days. The loss in weight initially showed that 10 g. of food/rat/day was not enough, although at first the rats would not eat more if it

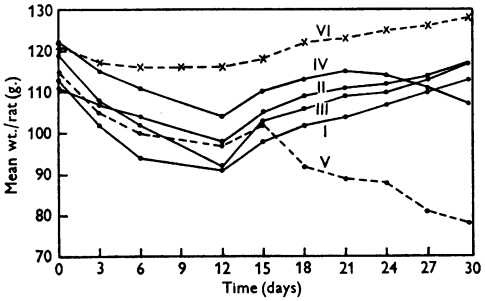

Fig. 1. Growth curves of rats receiving added bromobenzene (PhBr) and L-cystine in their diets (four rats in each group). The amounts of cystine and PhBr are expressed as g./100 g. of basal diet.

I, basal diet + 0·2 g. of cystine + 0·5 g. of PhBr;
II, basal diet + 0·4 g. of cystine + 1·0 g. of PhBr;
III, basal diet + 0·7 g. of cystine + 1·5 g. of PhBr;
IV, basal diet + 1·0 g. of cystine + 2·0 g. of PhBr;
V, basal diet, no cystine + 1·5 g. of PhBr;
VI, controls, basal diet alone.

contained bromobenzene, and on the seventh day they were given 14 g./day each. If the growth curves of groups I–V are compared with that of group VI which had also been clipped, but had a palatable diet from the beginning, then it is clear that the initial loss of weight is largely due to the lower intake of food. As mentioned earlier, after the 6th day the diet was mixed in another way and the rats ate more, and after the twelfth day all groups increased in weight. In groups I–III, this weight increase continued to the end of the experiment. In group IV the increase continued until the twenty-first day and then there was a gradual drop in weight. In group V, however, there was a slight increase for 3 days and then a pronounced loss in weight accompanied by the death of two of the animals in this group, the one on the twenty-first and the other on the twenty-seventh day of the experiment.

The observed changes in weights of the animal can be correlated with the output of mercapturic acid which is shown graphically in Fig. 2. During the first 6 days the percentage of the conjugate was low in all groups, and this corresponded with the period of loss of weight shown in Fig. 1. As the intake of food was low, the intake of bromobenzene was also low and, as suggested earlier, the incomplete mixing of the bromobenzene with the food allowed the rats to select particles of food free of bromobenzene. After the sixth day the output of mercapturic acid rose following better mixing of the bromobenzene with the diet. In groups I, II and III, the output of mercapturic acid tended to reach a steady level and a constant percentage of the dose was excreted as the conjugate. In the case of group IV, the constant percentage conjugation was reached at about the eighteenth to twentieth day, but thereafter there was a drop in the output which corresponded to the drop

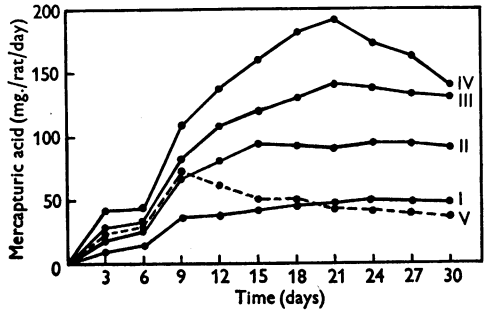

Fig. 2. Excretion of *p*-bromophenylmercapturic acid by rats receiving bromobenzene and L-cystine in varying amounts. For curves I–V, diets are as given in Table 1 and Fig. 1.

in weight of the animals as indicated in Fig. 1. The animals in group IV ate all their food until the twenty-eighth day; from the twenty-eighth to thirtieth day they left some of their food. With group V, there was the initial lag to the sixth day, followed by an increase of short duration and then a gradual fall in output after the ninth day. Here again the changes followed closely the growth curve in Fig. 1. The curve for group V should be compared with that for group III, for both these groups were receiving the same dose of bromobenzene, but the latter group received extra cystine as well. The percentages of bromobenzene excreted as mercapturic acid during the experiment are quoted in Table 6. According to Stekol (1943) the inhibition of growth of rats by bromobenzene is due to loss of cysteine as mercapturic acid. This lack of growth, however, can be prevented by addition of L-cystine to the diet. In the present experiments it appears that groups I–IV have sufficient cystine in their diets to cover the needs of growth, hair elaboration and the formation of mercapturic acid, because they gain weight (after the initial loss already explained), the cystine content

of their hair is normal, and the percentage of bromobenzene conjugated with cysteine is relatively constant. In group IV, however, there is a loss of weight and a drop in mercapturic acid production during the last 6 days of the experiment. This may be due to gradual poisoning by the very large dose of bromobenzene administered, or by the mercapturic acid, for Coombs & Hele (1927) have indicated that, in dogs, *p*-bromophenylmercapturic acid is much more toxic than bromobenzene. Group V seems definitely

Table 6. *Percentage of the dose of bromobenzene excreted as mercapturic acid*

	Percentage of dose excreted as mercapturic acid			
Group	Days 1–6	Days 7–12	Days 12–30	Average for the whole period
I	12	25·9	33	27
II	11	25·9	33	28
III	10·2	22·6	31	26
IV	10·6	21·6	31	25
V	9·0	15·8	12*	13†

* This figure is for twelfth to eighteenth days, because after the eighteenth day, all the food given was not eaten and therefore the amount of PhBr ingested could not be assessed.

† For 1–18 days.

to suffer from cystine deficiency as indicated by loss in weight; the cystine content of the hair is lower than in other groups and, although this group received the same amount of bromobenzene as group III, after the ninth day excretion of mercapturic acid was less than half the amount excreted by group III.

The distribution of bromine in the tissues examined (Table 3) showed that when bromobenzene is being fed there is an increase in the bromine content of the hair, muscle and liver. It is known that the bromine content of animal tissues varies with the concentration of the bromine of the diet (Winneck & Smith, 1937*b*). In groups I, II and III, where there is no apparent cystine deficiency, the bromine contents of the three tissues examined on the thirtieth day are of the same order, that of hair being slightly higher than liver and muscle. In group IV, the bromine content of the hair is appreciably higher than that of the first three groups, whereas in group V the hair bromine is nearly four times that of other groups. In all groups the bromine contents of the muscle and liver are of the same order. These figures suggest that where there is a cystine insufficiency brought about by bromobenzene feeding, the bromine appears in the hair in enhanced amounts. This further suggests that much of the available cystine in group V and some of it in group IV is being used for conjugation with bromobenzene and that perhaps some *S*-*p*-bromophenylcysteine is being used for elaboration of hair owing to shortage of cystine. The shortage of cystine is apparent in the analyses of hair given in Table 5, which shows that the hair of group V contains less cystine than that of other groups. If it is assumed that the bromine of the hair is present as *S*-*p*-bromophenylcysteine, $BrC_6H_4SCH_2CH(NH_2)COOH$, then it can be calculated that the amount of this compound appearing in the hair of each group on the thirtieth day is I, 33; II, 40; III, 38; IV, 52; and V, 133 mg./100 g. of dry defatted hair. In group V this would amount to about 1 % of the cystine present. However, the presence of *S*-*p*-bromophenylcysteine in the hair of these animals has not yet been proved. When bromobenzene is withdrawn from the diet as in the case of the surviving rat in each group which was kept until the forty-fourth day, the bromine content of the liver and muscles tends to return to the normal values obtained on the control group VI. In the case of the hair, however, the bromine content still rises in all groups. This is presumably due to residual bromine in the root which has grown out by the forty-fourth day.

The conclusion which can be drawn from these experiments is that in order to drive bromobenzene into the hair, in quantities which might be considered as 'therapeutic', a state of cystine insufficiency must be produced.

SUMMARY

1. A study has been made of the influence of varying amounts of bromobenzene and L-cystine added to the diet, on the growth, the bromine content of the hair muscle and liver, and the cystine content of the hair of young rats.

2. When the cystine content of the diet was adequate, the animals, when given bromobenzene, excreted a fairly constant amount of mercapturic acid each day, the cystine content of the hair was normal and there was no final loss in weight.

3. When the cystine of the diet was inadequate, bromobenzene feeding caused the animals to lose weight and to excrete less mercapturic acid than animals with adequate cystine. The cystine content of the hair was lower than normal.

4. On feeding bromobenzene with adequate cystine intakes, the bromine contents of the hair, muscle and liver rise above normal to approximately the same extent. On inadequate cystine intakes, the bromine content of the hair is much higher than that of the liver and muscles.

5. The suggestion is made that when bromobenzene is fed to rats on diets containing insufficient cystine for both growth and detoxication, some bromophenylcysteine is incorporated in the hair in an attempt to make good the lack of cystine.

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The Intermediary Metabolism of the Mammary Gland

3. ACETATE METABOLISM OF LACTATING MAMMARY GLAND SLICES WITH SPECIAL REFERENCE TO MILK FAT SYNTHESIS

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The origin of milk fat has been studied by various techniques (see review by Folley, 1949), the most favoured hitherto being the arterio-venous (A.V.) method which, however, can give little insight into the synthetic mechanisms involved. Arterio-venous studies have disproved the earlier idea of the utilization of blood phospholipin fatty acids for milk fat synthesis and have provided evidence that fatty acids of neutral fat, probably belonging to the glyceride fraction, are absorbed from the blood by the lactating udder, and are there transformed into glycerides of milk fat (see reviews by Folley, 1940, 1949).

Because they obtained a high respiratory quotient (R.Q.) by the A.V. method for the lactating goat udder, Graham, Houchin, Peterson & Turner (1938) postulated the formation of fat from carbohydrate by the lactating mammary gland (see also Reineke, Stonecipher & Turner, 1941). In apparent agreement with this finding, which had meanwhile been confirmed for the cow by other workers (see Folley, 1949), were the *in vitro* results of Folley & French (1948*a*, *b*, 1949*c*), who showed that lactating mammary slices from non-ruminants (mouse, rat, rabbit and guinea pig) utilize glucose *in vitro* with R.Q. > 1, results which were also interpreted as indicating the synthesis of fat from carbohydrate. The situation was, however, complicated by the fact that mammary gland slices from ruminants (cow and goat) were found to be practically inert towards glucose *in vitro*. As far as ruminants are concerned, therefore, these slice experiments provided no evidence to support *in vivo* indications of fat synthesis from carbohydrate, and threw little light on the nature of the precursors of milk fat.

Tracer studies have provided evidence of the utilization of acetate for fatty-acid synthesis in the mammal both *in vivo* (Rittenberg & Bloch, 1945) and in liver slices *in vitro* (Bloch & Kramer, 1948). Moreover, it is now known that in ruminants large quantities of acetic acid are produced in the rumen by fermentation of cellulose (Elsden & Phillipson, 1948) and that this acetate can be absorbed, leading to appreciable arterial blood levels (Reid, 1950, for sheep; McClymont, 1949, for cow). The possibility, arising mainly from the first of these tracer studies, that acetate is utilized by the mammary gland for milk fat synthesis was suggested by Folley (1945).

This paper deals with the acetate metabolism of mammary gland slices from various species, and presents evidence for the utilization of acetate for milk-fat synthesis by the lactating mammary gland. Part of this work has already been reported in preliminary communications (Folley & French, 1948*c*, 1949*a*, *b*) in the first two of which attention was drawn to the significance of these results for the origin of the short-chain fatty acids, which, as shown principally by Hilditch and his co-workers (Hilditch, 1947), are a prominent feature of the milk fat of herbivorous animals (although absent from body fat), and the origin of which has been the subject of controversy (see discussion by Folley, 1949). If acetate is utilized for milk-fat formation in the mammary gland, it seems probable that the short-