The sodium salt ('Indicator *R*-Na') (I; X=Na) is obtained by dissolving the acid in water (1700 ml.), at 60°, containing a slight excess of Na_2CO_3, salting out with NaCl (50 g.) and filtering when cold. Traces of impurity may be removed, if desired, by extraction of the dry salt with petroleum ether, b.p. 80–100°. Yield: 43 g.

Separation of acetyl-phenylalanine and acetyl-leucine

For the trial separations synthetic mixtures of acetyl-phenylalanine (1·84 mg.) and acetyl-leucine (2·06 mg.) were used. Columns were made up, as described by Gordon *et al.* (1943*a*), with 3·0 g. of silica gel, 1·4 ml. of a 0·025% aqueous solution of 'Indicator *R*-NH_4', and 2% butanol-chloroform. The chromatograms were developed with 2% butanol-chloroform and the evaporated fractions titrated with 0·0114N-$Ba(OH)_2$. The titre of the original mixture of acetylamino-acids was 1·828, 1·830 ml. The results obtained are shown in Table 1.

SUMMARY

1. The preparation of a new indicator, the ammonium salt of 3:6-disulpho-β-naphthalene-azo-*N*-phenyl-α-naphthylamine, is described.

2. This indicator is recommended for use in partition chromatography; its advantages for this purpose are discussed and examples of its use are given.

We are indebted to the Director General of Scientific Research and Development, Ministry of Supply, for permission to publish this paper.

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The Metabolism of 2:4:6-trinitrotoluene (α-T.N.T.)

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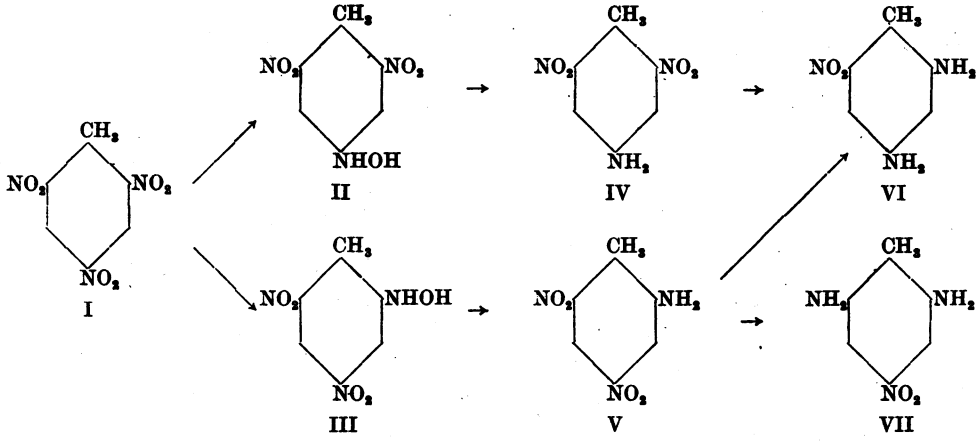
During the last war it was realized that prolonged exposure to T.N.T. might, in some cases, have serious effects on the health of workers in shell-filling factories, and much experimental work was therefore carried out on animals in order to throw light on the many problems presented by this observation. On the chemical side the information which resulted was, however, disappointing. Thus Dale (1921) encountered great difficulty in seeking to isolate and identify the metabolic products present in the urine of rabbits which had collectively received 13·2 g. T.N.T. The two crystalline products which he obtained represented no more than 7% of the T.N.T. administered; and while the identity of one, tetranitroazoxytoluene, was established, that of the other, believed to be a dinitroaminotoluene, was left in doubt. We have therefore sought to obtain further information on the difficult problem of the fate of T.N.T. in the body.

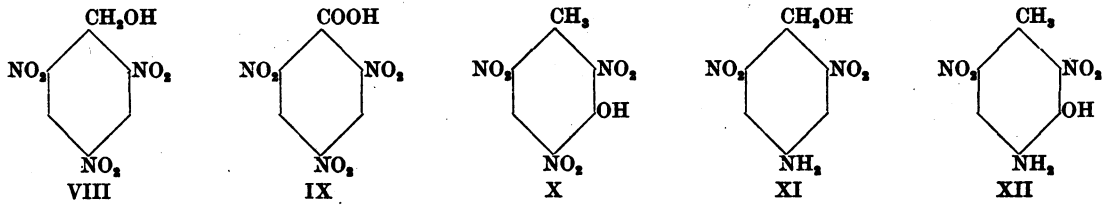
While only scanty studies have been made of the metabolic fate even of simple aromatic nitro compounds, it is possible to formulate a series of products which may arise when T.N.T. is administered to animals. Of the reduction processes, the more probable ones are the reduction of a single nitro group to an amino group, possibly through the intermediate stage of a hydroxylamino compound. Thus α-T.N.T. (I) might give rise either to 2:6-dinitro-4-hydroxylaminotoluene (II) or to its isomer, 2:4-dinitro-6-hydroxylaminotoluene (III), each of these compounds then undergoing further reduction to yield 2:6-dinitro-4-aminotoluene (IV) or 2:4-dinitro-6-aminotoluene (V); the dinitro-aminotoluenes might be similarly further reduced to the nitrodiaminotoluenes (VI, VII). Of the oxidation processes, the likely ones are those in which the CH_3 group has been oxidized to CH_2OH or COOH to give trinitrobenzyl alcohol (VIII) and trinitrobenzoic acid (IX), or the formation of trinitro-*m*-cresol (X) by introduction of a phenolic hydroxyl into position 3 of the T.N.T. molecule. Nor can the possibility of simultaneous oxidation and reduction with the production of such compounds as 2:6-dinitro-4-aminobenzyl alcohol (XI) and 2:6-dinitro-4-amino-*m*-cresol (XII) be eliminated.

The excretion of any of the products (I)–(XII) as such, is, however, not the whole story, for some of



them are likely to appear also in conjugated form coupled with glucuronic, acetic, sulphuric or other acids. Thus the amino compounds might appear as acetyl derivatives; (VIII), (IX), (X), (XI) and (XII) as glucuronides; trinitrobenzoic acid (IX) might appear as a substituted hippuric acid and (X) and (XII) as ethereal sulphates.

Webster test in which the T.N.T. compound is treated with KOH and alcohol, and we have applied it to a considerable number of possible T.N.T. metabolites and their derivatives. It has proved so valuable in guiding our chemical investigations that it is convenient to describe it here since it will be frequently referred to in the subsequent reading,



We record the formulae of these possible metabolites here, since they are frequently referred to in the subsequent reading. They illustrate the complexity of the problem, which is rendered more difficult by the similar solubilities possessed by compounds of such closely related chemical structure.

RESULTS

Since the work which we have carried out falls into a number of distinct parts, we propose first to describe the results of each with brief reference to the methods used. The findings will then be correlated in a later discussion, while the details of the experimental work and the synthesis of the reference compounds are presented at the end. All melting-points quoted are uncorrected.

(1) *The Cumming reactions of T.N.T. and some related compoinds*

Prof. W. M. Cumming very kindly put at our disposal a colour reaction which makes it possible to differentiate 2:6-dinitro-4-hydroxylaminotoluene and 2:2′:6:6′-tetranitro-4:4′-azoxytoluene. This colour reaction is a modification of the original

where it will be described as the Cumming reaction. Experience showed that the test was more valuable if it was carried out under approximately quantitative conditions. Our method has been to dissolve the substance in acetone so that its concentration was 0·1 mg./ml.; 1 ml. of the solution was then evaporated to dryness *in vacuo* and the residue dissolved in 1 ml. of a mixture of equal volumes of purified methyl ethyl ketone and *cyclo*hexanone; 0·5 ml. of 10% KOH was then added and the tube vigorously shaken; after standing some minutes the colour of the ketone layer was observed. Table 1 records the results.

(2) *Investigations of the material obtained by ether extraction of T.N.T. urine*

(*a*) *Preparation of the ethereal extract of T.N.T. rabbit urine and its content of T.N.T. metabolites.* We confirmed the findings of the workers of the last war that ether will extract little or no material from T.N.T. urine unless the urine, which is neutral or slightly alkaline in reaction when voided, is first acidified. When the acidified urine is extracted to exhaustion with ether in a continuous extractor, the

Table 1. *The Cumming reaction*

Compound	Colour
2:4:6-Trinitrotoluene (α-T.N.T.)	Red
2:3:4-Trinitrotoluene (β-T.N.T.)	Pale yellow
2:4:5-Trinitrotoluene (γ-T.N.T.)	Yellow
2:4:6-Trinitrobenzoic acid	Dull red
2:4:6-Trinitrobenzyl alcohol	Bright red
2:4:6-Trinitrobenzyl acetate	Bright red
2:4:6-Trinitrobenzyl methyl ether	Bright red
1:3:5-Trinitrobenzene	Dull red
2:4-Dinitrotoluene	Purple
m-Dinitrobenzene	Reddish purple
2:6-Dinitro-4-hydroxylaminotoluene	Brownish red
N-(3:5-dinitro-4-methylphenyl)-*iso*benzaldoxime	No colour at first, then gradually becoming pink and finally brownish red
2:6-Dinitro-4-aminotoluene	No colour } Green in concentrated solutions
2:4-Dinitro-6-aminotoluene	No colour }
2-Nitro-4:6-diaminotoluene	No colour
2:2':6:6'-Tetranitro-4:4'-azoxytoluene	Deep blue
2:4:6-Trinitro-*m*-cresol	Yellow
2:6-Dinitro-4-amino-*m*-cresol	Orange
Trinitromesitylene	No colour

extract, dried over anhydrous sodium sulphate, yields on evaporation a viscous brown tar in amount corresponding to 88 mg./kg. of rabbit. That the bulk of this material has no relation to T.N.T. metabolism is shown by the fact that a product similar in appearance and in yield corresponding to 61 mg./kg. of rabbit is obtained from the control animals. The difference in these yields suggests that of the 150 mg. T.N.T./kg. administered, 27 mg. or 18 % has appeared in the ethereal extract. We obtained confirmatory evidence on this point in two ways. First, we were finally able to remove much extraneous material from these intractable tars by pouring their very dilute acetone solutions slowly into ten volumes of vigorously shaken water. The suspensions, which were acid, were made just alkaline with Na_2CO_3 and then extracted with ether. The yield obtained from the ethereal extract of the T.N.T. tar was 28·3 mg./kg. rabbit, that from the control tar 5·5 mg./kg.; the difference of 22·8 mg./kg. corresponds to 15·2 % of the T.N.T. administered. Secondly, in some preliminary chromatographic experiments, we purified each tar by pouring its 10 % solution in acetone into ten volumes of benzene. The material remaining soluble was obtained by evaporation of the solvent, and, after solution in dry benzene, was passed through an alumina column. The total weight of material obtained from the combined eluates corresponded in the case of the T.N.T. tar to 31·7 mg./kg. rabbit, and for the control tar to 9·8 mg., the difference, 21·9 mg., corresponding to 14·6 % of the T.N.T. administered.

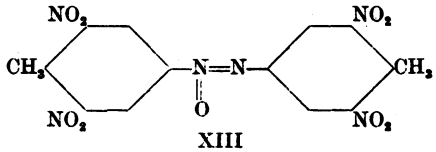
Since these very different methods of assessing the amount of T.N.T. products extractable from the urine by ether give very similar results, 18·0, 15·2 and 14·6 %, we regard it as a reasonable conclusion that no more than 15 % of the T.N.T. administered is excreted as compounds soluble in ether, for although this extract was obtained only from the urine secreted in the 24 hr. following administration, indirect evidence mentioned later shows that small doses of T.N.T., such as those under discussion, are almost completely eliminated in that time; further, the urine of the second day, treated as just described, can be shown to contain only very small amounts of ether-soluble T.N.T. substances.

While this 15 % may possibly contain small amounts of acetylated amino derivatives, it is likely to contain all the metabolic products which are excreted in unconjugated form, for conjugation with such compounds as glucuronic and sulphuric acids will almost certainly result in compounds which are insoluble in ether. If this deduction be accurate, some 85 % of the T.N.T. is excreted as conjugated compounds of various types. The purified ether-soluble material gave a strong purple Cumming reaction and a strongly positive diazo reaction for aromatic amino groups, in which we used alcoholic dimethyl-α-naphthylamine as the coupling reagent.

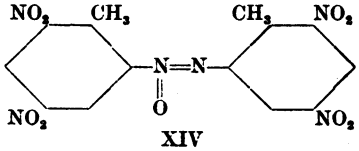
(*b*) *Chromatographic analysis.* Study of the solubilities of the numerous possible metabolites which we had synthesized showed that with very few exceptions they shared common solubilities in solvents which are far from ideal for use in chromatographic separation. After much preliminary work, the main bulk of the T.N.T. material, after initial purification through the acetone-water-ether method mentioned above, was finally dissolved in dry benzene and passed through an alumina column. While elution of the column proved tedious, the application of the Cumming and diazo reactions to the fractions showed that good separation had been achieved in the earlier fractions. Thus the first five fractions gave a negative diazo reaction, while the next six gave a negative Cumming reaction; the

sixty-five fractions were therefore suitably recombined on the basis of their colour reactions to give five fractions A–E. Further purification of these through chromatography having proved unsuccessful, we proceeded to their direct chemical investigation.

(*c*) *The azoxy compounds present in* T.N.T. *urine.* Fraction A, an orange wax, gave an intense purplish-blue Cumming reaction and, since its diazo reaction was negative, we concluded that the fraction consisted mainly of azoxy compounds. Without difficulty 26 % of it was isolated as pure crystalline 2:2′:6:6′-tetranitro-4:4′-azoxy-toluene (XIII).



XIII



XIV

The isolation of this compound from T.N.T. urine is not new, for it has been previously obtained from the urine of rabbits (Dale, 1921), of workers in T.N.T. factories (Moore, 1917; Webster, 1921) and of rabbits and monkeys (Moore, 1917). The question as to whether it is formed *in vivo*, however, needs discussion. Moore (1917), realizing that azoxy compounds can be readily formed from the corresponding hydroxylamines, suggested that the substance actually excreted might be a hydroxylamine from which the azoxy compound arose as a result of the procedures used in its isolation. Accordingly, Webster (1921) endeavoured to isolate a hydroxylamine, but meeting with no success, concluded that the azoxy compound was excreted as such. A similar conclusion was reached by Dale (1921) who, approaching the problem from the other end, stressed the fact that he had now obtained the azoxy compound without subjecting the extract to any treatment which, as he thought, could lead to its formation from a pre-existing hydroxylamino compound. The *in vivo* formation of the azoxy compound thus seemed established. The striking insolubility of this azoxy compound in almost all solvents and the novel form of the reduction process entailed in its direct production from T.N.T. led us, however, to enquire further into this matter. We found that, after the necessary acidification, both the urine of rabbits receiving T.N.T. and the ethereal extract prepared from it gave the Cumming reaction for the hydroxylamine only. Bearing in mind that the Cumming reaction for the hydroxylamine in very dilute solutions is pale pink, while in the case of the azoxy compound the reaction is particularly sensitive and results in an intense blue colour, this was, in our opinion, proof enough of the absence of the azoxy compound from freshly voided urine. This conclusion was confirmed by our finding that the material obtained by evaporation of the ether now gave the blue Cumming reaction for the azoxy compound, and proving that the azoxy compound was also produced during the procedures used by us to prepare our materials for chromatographic separation. Lastly, as will be seen later, it proved possible to isolate from rabbit urine the dinitrohydroxylaminotoluene. The azoxy compound is, therefore, an artefact and does not pre-exist in the urine. There is thus no evidence for the *in vivo* formation of azoxy compounds, for Sieberg (1915) pointed out that the azoxybenzene detected by Lewin (1895) in the urine of a chemist poisoned while preparing β-phenylhydroxylamine, and by Mayer (1905) in the urine of a rabbit given phenylhydroxylamine, could have been readily formed by exposing to air urine containing the parent substance.

From the remainder of fraction A there were obtained traces of an orange compound which resembled the 4:4′-azoxy compound in giving a blue Cumming reaction, a negative diazo reaction and in being characteristically insoluble in most organic solvents. It differed, however, in two respects: its m.p. 165–167° contrasted strongly with the m.p. 215° of the 4:4′-compound, while its colour was orange as compared with the creamy white of the latter. The amount available did not permit of further investigation, but for reasons which will appear later we think it possible that this orange compound is the 2:2′:4:4′-tetranitro-6:6′-azoxy-toluene (XIV), although it could be the geometrical isomeride of the 4:4′-azoxy compound, since azoxy compounds occur in *cis* and *trans* forms (Sidgwick, 1942). Unfortunately, the 6:6′-azoxy compound is not described in the literature and, although Brand & Eisenmenger (1913) have described a compound, m.p. 109°, which they claim to be the 6-hydroxylamine, we were unable to repeat their preparation; all our attempts to prepare these compounds have, so far, failed. Hence this point must remain undecided for the time being.

Two further comments on this fraction are desirable. First, it must be emphasized that little more than 25 % of it has been isolated as azoxy compounds, in spite of the insolubility of the latter in the pure state. That azoxy compounds were still present in the residue in significant amounts was clear from the Cumming reaction, and the solubility is doubtless affected by the presence of other substances. Secondly, it will be recalled that the

original fraction gave a purple Cumming reaction in contrast to the deep blue given by the pure azoxy compound. We interpreted this as meaning that a substance giving a red Cumming reaction was also present, and we observed that as the azoxy compound was progressively removed the purple Cumming reaction of the residue became increasingly reddish in hue. The only substances giving a red Cumming reaction which are likely to be present are 2:4:6-trinitrobenzyl alcohol, 2:4:6-trinitrobenzoic acid or 2:4:6-trinitrobenzene, for at no point in this work have we observed the excretion of unchanged T.N.T. In spite of persistent effort we have, however, failed to isolate any of these substances or any other compound from what is the major part of this fraction. This point will be referred to again.

(*d*) *The dinitroaminotoluenes.* That there is present in T.N.T. urine a substance which is probably a dinitroaminotoluene is already known. Its identity has, however, yet to be established. Thus Webster (1921) showed by mixed m.p. determinations that the yellow-brown substance, m.p. 162°, isolated by him from the urine of T.N.T. workers, was not identical with authentic 2:6-dinitro-4-aminotoluene, m.p. 171°. Likewise, the product obtained by Dale (1921) from T.N.T. rabbit urine had an initial m.p. 154–155°, raised to 171° by recrystallization, but this recrystallized product also considerably depressed the m.p. of pure 2:6-dinitro-4-aminotoluene. Dale commented on the doubts which exist concerning the m.p. of the other isomer, 2:4-dinitro-6-aminotoluene, and was forced to leave the question of the identity of his product open. Our own experiences in the attempted synthesis of these two isomers confirmed those of Dale and made it necessary to investigate this chemical question in detail before treatment of our biological material.

There is no doubt concerning the authenticity of 2:6-dinitro-4-aminotoluene, for it has been prepared by the reduction of α-T.N.T. with H_2S in the presence of ammonia by Hollemann & Boeseken (1897), by Cohen & Dakin (1902) and by Anschütz & Zimmermann (1915); by electrolytic reduction of T.N.T. by Hofer & Jacob (1908) and by Brand & Eisenmenger (1913), and the latter workers also prepared it from the corresponding hydroxylamine compound. Its recorded m.p. is 171°.

The position regarding 2:4-dinitro-6-aminotoluene is, however, extremely unsatisfactory. The m.p. of this substance is given by Hollemann & Boeseken (1897) and by Anschütz & Zimmermann (1915) as 155°. However, Dale (1921), who repeated the preparation of the former workers, found that the m.p. of their product could be raised by recrystallization from the published figure of 155° to 171°, and he decided that their preparation was merely an impure specimen of the 4-amino compound; Dale reported also the failure of all his own attempts to prepare the 6-isomer. The literature is further confused in that Brand & Eisenmenger (1913) report that the m.p. of the 6-amino compound, prepared by electrolysis of T.N.T., is not, as claimed by Hollemann & Boeseken, 155°, but is 135°, and they later supported their finding by showing that their product on diazotization and boiling in ethanol yields 2:4-dinitrotoluene and 2:4-dinitroindazole, compounds of which the identity could not be in doubt.

We have repeated Brand & Eisenmenger's electrolytic reduction of T.N.T. and have isolated their compound, m.p. 135°. The various preparations melted from 130 to 135°, and while little change is effected by recrystallization from water, the m.p. can be raised to 135–140° when benzene is employed. These unsatisfactory m.p.'s led us to investigate further, and it was found that the acetyl derivative prepared from the Brand & Eisenmenger product and recrystallized from ethanol had m.p. 227°, which is that of an authentic specimen of the acetyl derivative of the wrong isomer. If, therefore, the Brand & Eisenmenger product contained the 6-isomer, it seemed likely that it was a mixture. That this was so was proved by investigation of the benzoyl derivative, which, as first prepared, has m.p. 220–230°. By fractional crystallization from ethanol, the benzoyl derivative can be made to yield with difficulty two compounds of sharp m.p.: one, identical with authentic 2:6-*dinitro*-4-*benzamidotoluene*, which crystallizes in plates, has m.p. 259–261°; the other, which appears in needles, m.p. 216–217°, was later shown to be 2:4-*dinitro*-6-*benzamidotoluene*. These results show that the 6-amino compound has never been prepared in a pure condition.

Since the fractionation of the benzoyl derivatives was extremely wasteful and their debenzoylation difficult, it was desirable to seek a more satisfactory method for application to the separation of our urinary products. This was achieved by the finding that in the presence of excess benzenesulphonyl chloride and pyridine, the 4-amino compound forms an ethanol-soluble *mono*benzenesulphonyl derivative, whereas the 6-amino compound forms an alcohol-insoluble *di*benzenesulphonyl compound. The free dinitroaminotoluenes can be readily recovered from these compounds by hydrolysis with 80% (v/v) H_2SO_4. 2:4-*Dinitro*-6-*aminotoluene* prepared in this way forms yellow needles, m.p. 176°; the 4-isomer is orange and has m.p. 174°. The m.p. of a mixture of the two isomers is 135–150°, which is that of the Brand & Eisenmenger preparation. The acetyl derivative obtained by the latter workers, m.p. 224°, is, as we have indicated, that of the 4-isomer; that of the 6-amino compound prepared from the pure base has m.p. 159–160°.

In order to clarify the position, the essential findings are brought together (Table 2).

Table 2. *Melting-points*

Derivative	2:6-Dinitro-4-aminotoluene	2:4-Dinitro-6-aminotoluene
Free base	174°	176°
Acetyl-	227°	159–160°
Benzoyl-	263–264°	216–217°
Benzenesulphonyl-	(mono) 177–178°	(di) 222°

We now return to fraction B of the urinary material. The intense diazo reaction given by this fraction, its Cumming reaction negative under our standard conditions but green when more material was used for the test, the ease with which it was almost quantitatively recrystallized from water as a golden yellow crystalline mass, and its elementary analysis showed that it could contain little but dinitroaminotoluenes. Before the chemical difficulties just described had been appreciated, the fraction had been separated, by recrystallization from water, into three fractions, B_1, 1·03 g., m.p. 129–131°; B_2, 0·39 g., m.p. 140°; B_3, 0·41 g., m.p. 129–134°. By application of the benzenesulphonyl separation to B_1, the presence of both the 4-amino and the 6-amino compound was established, while the weights of the two benzenesulphonyl derivatives obtained indicated that 70% of the fraction consisted of the 4-amino compound and the remainder of the 6-amino compound. The range of m.p.'s of fractions B_2 and B_3 had already been encountered in the chemical work just described, and as no qualitative difference between any of the fractions was demonstrable, it was considered unnecessary to apply the benzenesulphonyl separation to them. For the calculation of yields it was therefore regarded as sufficiently accurate to deduce that fraction B contained about 1·4 g. 2:6-dinitro-4-aminotoluene and 0·6 g. 2:4-dinitro-6-aminotoluene.

Fraction C gave a weak purple Cumming reaction, a strong diazo reaction and feebly reduced Benedict's reagent, indicating that it was a mixture of dinitroaminotoluene, azoxy compounds and possibly traces of hydroxylamine. By extraction with boiling water, the azoxy compounds were separated and the water extract yielded two new fractions, C_1, 0·26 g., and C_2, 0·15 g. C_1 was dinitroaminotoluene similar to the unresolved B fraction, for its m.p. was 132–134°, its Cumming reaction was negative and its diazo reaction strongly positive. The fact that C_2 gave a feeble Cumming reaction for the hydroxylamine and was also feebly reducing to Benedict's reagent showed that such hydroxylamine as remained unconverted to azoxy compound had been concentrated into it. By far the greater proportion of it was, as shown by the intensity of its diazo reaction and its m.p. of 128°, also dinitroaminotoluene, and attempts to isolate the traces of hydroxylamine failed. The residue from these fractions was a brownish-red glass from which nothing crystalline was obtained; its colour reactions indicated that it was probably a mixture of amino, hydroxylamino and azoxy compounds.

No positive information was obtained regarding the nature of the small fraction D which had been obtained by elution of the column with benzene : ether, 3 : 1, save that colour tests indicated that it might contain traces of hydroxylamine. The larger fraction E, which represented the combined eluates from the column after the application of ethanol : ether, 3 : 1 and ethanol-HCl, was a dark brown semi-solid, which appeared to contain little, if any, T.N.T. metabolite; it was not further investigated.

(3) *The isolation of 2:6-dinitro-4-hydroxylaminotoluene from T.N.T. urine*

The clear proof obtained that the tetranitroazoxytoluene was absent from freshly voided urine made it reasonably certain that it must have arisen from a dinitrohydroxylaminotoluene. Attempts to isolate the latter proved very difficult because of the ease with which it is oxidized to the azoxy compound. The final method adopted was to extract the urine for 3 hr. only with peroxide-free ether, and to heat the dry residue, obtained after careful evaporation of the ether, with benzaldehyde for 1–2 min. From the mixture so obtained, there was isolated *N*-(3:5-*dinitro*-4-*methylphenyl*)-iso*benzaldoxime*, identical with an authentic specimen. While such an ethereal extract contains dinitroaminotoluenes, it was possible to show by a separate experiment that the amino compounds do not condense with benzaldehyde under the conditions used, and they could therefore be readily separated from the hydroxylamine. The highest yield of purified oxime obtained was 10 mg./g. T.N.T. administered. This yield is certainly much less than the amount of hydroxylamine present because of the great ease with which the latter is converted to the azoxy compound.

Having established the chemical nature of the uncombined T.N.T. metabolites, we turned to investigate the conjugated products. Experience soon showed that these investigations were unlikely to prove fruitful until some indications as to the type of product likely to be encountered had been first obtained. The remainder of this paper is therefore concerned with indirect observations directed to this end.

(4) *The estimation of dinitroaminotoluene in T.N.T. urine*

Having established that the 4-amino compound was the major constituent of the dinitroaminotoluene of the urine it was decided that valuable

quantitative information might be obtained if this substance were used for the estimation of the total T.N.T. excretion products which contained an NH_2 group. The method used was similar in principle to that employed for the estimation of sulphanilamide in urine. The amino compound was diazotized and coupled with *N*-(1-naphthyl)-ethylenediamine and the resulting red colour was measured with a 'Spekker' photoelectric absorptiometer with an Ilford No. 602 Blue Filter, the red dye being estimated in 50 % (v/v) aqueous ethanol since it tended to precipitate from aqueous solution. The results which appear in Table 3 were then read from a standard curve constructed from estimations carried out on a solution of 2:6-dinitro-4-aminotoluene in normal rabbit urine. Table 3 shows first that on the wet diet, which resulted in a urinary volume of 200 ml., 29·7 % of the T.N.T. is excreted as amino compounds; on the dry diet, with a urinary volume only 50 ml., the figure is 24·2 %. Whether the difference in these figures is significant, is doubtful. Secondly, estimations following hydrolysis of the urine show, in all cases, an increase in estimable amino compounds of the approximate order of 10 % of the T.N.T. fed. This might be due either to the liberation of amino groups by hydrolysis of acetylated amino compounds or to the conversion of hydroxylamino compounds to their corresponding amino compounds. Since we have yet to encounter acetylated amino compounds in T.N.T. urine, while we have established the presence of hydroxylamine, we think the latter explanation much more probable. In this connexion, mention is necessary of the fact that hydroxylamines themselves react slightly to give a weak diazo reaction. In view of the preponderance of dinitroaminotoluenes this will not seriously influence the values obtained on unhydrolyzed urine; nor will it invalidate the increase which follows hydrolysis. Thirdly, the proportion of the T.N.T. excreted as amino compounds is unaffected by the amount administered.

(5) *The excretion of glucuronic acid and ethereal sulphates*

The fact that the uncombined T.N.T. compounds which we had isolated were apparently present in such small amounts led us to study quantitatively the glucuronic acid excretion. The results appear in Table 4, where in column 6 the extra glucuronic acid caused by T.N.T. administration is calculated in terms of T.N.T., on the assumption that no T.N.T. metabolite is linked with more than one molecule of glucuronic acid. This table shows (*a*) that an average of 48 % of the T.N.T. is excreted in combination with glucuronic acid, (*b*) that the total excretion of glucuronides is both independent of the dose, and uninfluenced by the different urinary volumes resulting from the 'wet' and 'dry' diets. Study of the daily excretion of individual rabbits shows that on doses of T.N.T. of the order of 200 mg./kg. or less, glucuronide excretion is complete within 24 hr.; with higher doses, 48 hr. may be required. This is illustrated by a few typical examples given in Table 5, which also shows not only the range of variability encountered in the glucuronic acid excretion of individual rabbits from day to day, but also that the amount of glucuronic acid excreted may vary significantly from rabbit to rabbit even though the diet be constant. These findings as to the rate of excretion of T.N.T. in relation to the size of the dose are confirmed by the figures recorded on p. 84 concerning the rate of excretion of amino compounds.

Table 3. *T.N.T. reduction products present in rabbit urine*

		Dose of T.N.T.		Reduction products: As dinitro-4-aminotoluene (mg.)		Reduction products: As T.N.T. (% of dose administered)	
Diet	Rabbit	(mg.)	(mg./kg. of rabbit)	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
Wet	1	170	103	37·2		25·2	
	13	230	105	55·3	80·3	27·6	40·0
	2	330	206	106·2		37·0	
	48	420	210	129·5	154·5	35·4	42·3
	15	675	321	144·8		24·6	
	39	585	344	143·9		28·3	
					Mean	29·7	
Dry	31	225	100	51·6		26·3	
	37	170	92	41·2	49	28·5	33·3
	54	360	200	84·6		27·0	
	61	430	200	89·4	123·5	23·9	33·0
	20	570	300	84·6		17·2	
	29	750	306	148·1		22·6	
					Mean	24·25	

Table 4. *Increased output of glucuronic acid caused by T.N.T.*

Diet	Rabbit	Dose of T.N.T. (mg.)	Dose of T.N.T. (mg./kg. rabbit)	Increased glucuronic acid output calculated: As glucuronic acid (mg.)	As T.N.T. (mg.)	As percentage of administered T.N.T.
Wet	12	98	50	53	61	63
	18	99	50	49	57	58
	23	200	100	93	108	54
	28	204	100	76	88	44
	44	285	150	94	109	39
	47	278	150	97	112	41
	40	450	200	202	234	52
	41	400	200	138	160	40
	51	500	250	264	306	62
	62	525	250	149	172	33
	12	585	300	149	172	30
	18	615	300	245	284	47
	23	753	350	274	317	43
	28	753	350	298	345	46
	50	800	400	376	435	55
	62	841	400	386	447	54
	40	1013	450	463	536	53
	18	975	500	397	460	48
	23	1128	550	390	451	40
	28	1100	550	445	515	47
	40	1230	600	455	527	43
Dry	41	660	300	239	277	42
	44	718	350	339	462	65
	45	800	400	407	471	59
	51	1000	500	388	449	45
	67	1128	550	564	653	58
	43	1222	650	358	415	43

Table 5. *Glucuronic acid excretion of individual T.N.T.-treated rabbits*

Rabbit	Dose of T.N.T. (mg./kg.)	Glucuronic acid output (mg./day) during day: 1	2	3*	4	5	6
36	0	109	107	118	85	134	—
18	50	100	96	135	49	101	—
41	200	97	108	245	100	125	—
51	250	94	95	216	283	146	134
62	400	127	118	347	316	145	164
28	550	168	137	572	161	120	151

* T.N.T. administered on the third day.

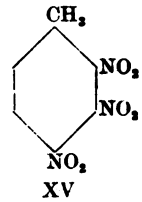
Repeated investigation showed that T.N.T. administration had no effect on ethereal sulphate excretion.

(6) *The red pigment of T.N.T. urine*

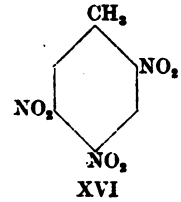
Webster (1921) noted that the urine of his experimental rats was 'a bright pink colour', and recently Himsworth & Glynn (1942) have shown that the intensity of the colour of the urine of rats receiving T.N.T. depends on the nature of their diet. The urine of our experimental rabbits during the first 6 hr. was orange-red in colour and the presence of the red pigment could be readily shown.

In view of the great difficulty of isolating the T.N.T. metabolites, it occurred to us that valuable indirect evidence on the chemical nature of the red pigment might be obtained by making observations on the colour of the urine secreted by rats to which various possible T.N.T. metabolites were administered. For this purpose individual rats receiving the usual stock diet were placed in small cages standing on a sheet of white filter paper, so that their urine soaked into the paper; this method made it possible to observe the formation and intensity of the red pigment with certainty.

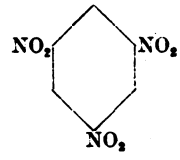
The compounds used were α-T.N.T. (I) and its two isomers 2:3:4-T.N.T. (β-T.N.T. XV), and 2:4:5-T.N.T. (γ-T.N.T. XVI); the reduction products already isolated, namely 2:6-dinitro-4-hydroxylaminotoluene (II), 2:6-dinitro-4-aminotoluene (IV) and 2:4-dinitro-6-aminotoluene (V), together with another possible reduction product, 6-nitro-2:4-diaminotoluene (VI).



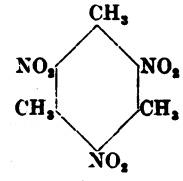
XV



XVI



XVII



XVIII

Of the oxidation products, 2:4:6-trinitrobenzyl alcohol (VIII), the presence of which we had already suspected from colour reactions given by fraction A, and the further substances which might arise from it, 2:4:6-trinitrobenzoic acid (IX) and 1:3:5-trinitrobenzene (XVII) were used. In order to obtain evidence as to whether the pigment arose through introduction of a phenolic hydroxyl into position 3, we administered two compounds: 2:4:6-trinitro-*m*-cresol (X), which has a hydroxyl in the required position, and trinitromesitylene (XVIII) in which the position is blocked by the methyl group, the latter being relatively resistant to biological attack.

Each compound was dissolved in olive oil and each rat received an amount of its particular compound molecularly equivalent to 150 mg. T.N.T./kg., either orally or by subcutaneous injection; the result obtained was found to be independent of the route of administration. Every experiment was controlled by injection of one animal with α-T.N.T.,

the colour of the resulting urine being used as a rough standard.

Of the compounds used, only α-T.N.T. and trinitrobenzyl alcohol caused red urines; in all the other cases, save trinitromesitylene which resulted in no colour, the urinary stains were various shades of yellow.

Two deductions were immediately possible. First, that red pigment formation resulting from commercial T.N.T. is not due to the presence in it of small amounts of the β- and γ-isomers. Secondly, that trinitrobenzyl alcohol may be a constituent part of the red pigment or at least an intermediate stage in its formation is suggested not only by the fact that it alone caused the production of a red stain, but also by the fact that the stain was the deep red colour of venous blood compared with the deep pink caused by T.N.T. itself. The necessity for the presence of the —CH_2OH group, shown by the lack of result with the closely related trinitrobenzoic acid, was further established by administering to other animals the methyl ether and the acetate of 2:4:6-trinitrobenzyl alcohol. We anticipated that the acetate would be partly hydrolyzed in the body with the production of a coloured urine; on the other hand, demethylation of the ether was likely to prove much more difficult, and little if any colour should be found in the urine. Such indeed proved to be the case. The urine from the animals receiving the acetate was less intense in colour than that of those receiving T.N.T. but was still bright pink; that from those receiving the ether was shell pink, and the trace of colour resulting might well have been due to the difficulty encountered in purifying the ether.

One further observation is worthy of record. After administering 2:4:6-trinitrobenzyl alcohol to rabbits, it was found that the resulting urine not only contained red pigment, but also gave an intense diazo reaction, indicating that one or more of the nitro groups had been reduced. It is therefore possible that the red pigment itself may result from a partial reduction product of the alcohol. From these experiments, we conclude that the red pigment present in the T.N.T. urine is probably a derivative of 2:4:6-trinitrobenzyl alcohol.

As to its nature we have no clear indication. These red urines are decolorized on acidification with mineral acid when they turn yellow. This suggests that the red pigment may be a salt, since it is known that many polynitro bodies form red or orange salts with alkalis, which are converted into yellow nitro substances on acidification. Webster (1921) pointed out that the urine of rats receiving T.N.T. may be a bright pink colour, even though the urine is quite acid (Himsworth & Glynn, 1942); this does not finally dispose of the matter, however, for the salt may be stable to some degree of acidity. In this connexion, it is worthy of mention that, although 2:4:6-trinitrobenzoic acid also gives a red salt with NaOH, its administration does not result in the formation of the red pigment.

DISCUSSION

The isolation of 2:6-dinitro-4-hydroxylaminotoluene and 2:6-dinitro-4-aminotoluene shows the existence of the reduction mechanism.

$$CH_3C_6H_2(NO_3)_3 \rightarrow CH_3C_6H_2(NO_2)_2NHOH \rightarrow CH_3C_6H_2(NO_2)_2NH_2.$$

Although the difficulties encountered prevented the isolation of the 2:4-dinitro-6-hydroxylaminotoluene, the isolation of its further reduction product, 2:4-dinitro-6-aminotoluene, makes it reasonably certain that the first step in the reduction of the 6-nitro group is also the production of the hydroxylamine.

In addition to undergoing reduction, T.N.T. may also be oxidized, for the results of the use of trinitrobenzyl alcohol in the experiments on the origin of the red pigment indicate the probable existence of the oxidation mechanism

$$CH_3C_6H_2(NO_3)_3 \rightarrow CH_2OH.C_6H_2(NO_2)_3.$$

Present knowledge of detoxication mechanisms suggests that the glucuronides of T.N.T. urine must arise from T.N.T. oxidation products, since glucuronic acid is found naturally combined only with compounds possessing an aliphatic or aromatic hydroxyl group or a carboxyl group attached to an aromatic ring. The only compounds to which T.N.T. can give rise which fulfil this condition are trinitrobenzyl alcohol, trinitrobenzoic acid and trinitro-*m*-cresol and products resulting from the reduction of one or more of the nitro groups of these three compounds. The fact that T.N.T. urines are non-reducing means that the glucuronides must be of the ether type, for ester glucuronides are reducing substances. Hence, trinitrobenzoyl glucuronides can be eliminated from discussion. As to the presence or absence of trinitro-*m*-cresol we have no certain information. The fact that administration of T.N.T. does not cause a rise in ethereal sulphate excretion might be taken as evidence of its absence, since an increase in ethereal sulphate usually indicates the presence of a compound containing a phenolic group. Such a deduction may be an uncertain one, however, because some phenols, such as salicylic acid, are excreted, in the rabbit, unconjugated with sulphate (Williams, 1938). It is worth mention here also that even if trinitro-*m*-cresol were present the fact that it contains two nitro groups *ortho* to the hydroxyl would be against conjugation occurring. While the position regarding trinitro-*m*-cresol must remain open for the present, that of the third substance, trinitrobenzyl alcohol, is much

more certain. The results of the experiments on the formation of the red pigment demonstrated not only that this alcohol may be produced from T.N.T., but also that it may be reduced *in vivo* to a diazotizable amino compound, presumably 2:6-dinitro-4-aminobenzyl alcohol. Either this compound or its parent substance could form ether glucuronides through their CH_2OH groups, and it seems reasonable to us tentatively to conclude that the glucuronides of T.N.T. urine are probably trinitrobenzylglucuronide or dinitroaminobenzylglucuronide or a mixture of both. The possible existence of the former is strengthened by the analogous case of *o*-nitrotoluene, for Jaffe (1878–9) proved this compound to be oxidized to *o*-nitrobenzyl alcohol, which is excreted as *o*-nitrobenzylglucuronide.

Since little material can be extracted from T.N.T. urine until the urine is acidified, the workers of the last war tended to interpret this finding as meaning that T.N.T. metabolites existed in the urine in union with glucuronic acid. Thus Panton (1921), Dale (1921), Webster (1921) suggested that their tetranitroazoxytoluene existed in this form, and Moore (1917) that his hypothetical hydroxylamine similarly existed. The azoxy compound contains neither the hydroxyl nor the carboxyl, either of which is necessary for glucuronide formation, and in any case we have shown this compound to be an artefact arising from the hydroxylamine; nor is there any analogy to support the suggestion of Moore that the dinitrohydroxylaminotoluene is combined with glucuronic acid, for no case is known of the attachment of a hydroxylamino group to a uronic acid. Whatever may be the explanation of the fact that T.N.T. compounds cannot be extracted from urine unless the urine is acidified, it must be emphasized that acidification of normal urine also causes much material to become extractable by ether; the fact that T.N.T. urine needs acidification before its T.N.T. metabolites can be removed by ether thus seems a poor argument on which to base the suggestion that these compounds are combined as glucuronides.

The isolation of the hydroxylamine is of interest for two reasons. First, while the production of hydroxylamino compounds as metabolites arising from both aromatic amino and nitro compounds has been postulated by several workers (e.g. Ellinger, 1920; Lipschitz, 1920; James, 1940), this is the first occasion on which a hydroxylamine has been isolated and its structure proved. Secondly, it is noteworthy because Wyon (1921) found that the hydroxylamine is more toxic than the parent T.N.T. Its isolation thus provides an example of the production of a more toxic product in a so-called detoxication mechanism. We have found the hydroxylamine to be a powerful methaemoglobin-former *in vitro*. It is very sparingly soluble in water (*c.* 0·05 %), and if its solution in water is added to an oxyhaemoglobin solution the spectrum of methaemoglobin becomes immediately visible (cf. Voegtlin, Hooper & Johnson, 1920). The two amino compounds have no effect on blood under the same conditions. Since the first stage in the biological reduction of T.N.T. is the formation of dinitrohydroxylaminotoluene, there is produced a very reactive compound which may be harmful to the blood and may have toxic effects on some of the enzyme systems. The hydroxylamine can be detoxicated by conversion into the relatively non-toxic dinitroaminotoluenes and its toxic effects will depend on the rate at which this conversion occurs. While the conversion takes place *in vivo* it is by no means complete, for the hydroxylamino and the amino compounds are excreted in the urine in roughly similar amounts. The formation of dinitrohydroxylaminotoluene may therefore partly explain the toxic action of T.N.T. Wyon (1921) showed that large doses of T.N.T. given to rats and rabbits cause the appearance of a chocolate-coloured pigment in the blood which he could not identify with certainty as methaemoglobin. It is possible that there may be formed in the blood from dinitrohydroxylaminotoluene pigments similar to those described by Keilin & Hartree (1943) from phenylhydroxylamine and methaemoglobin and other blood pigments.

We have also observed that trinitrobenzyl alcohol is also a fairly potent methaemoglobin-former *in vitro*. On adding aqueous solutions of the alcohol, which is sparingly soluble in water, to an oxyhaemoglobin solution, the spectrum of methaemoglobin becomes visible in less than half an hour. The action of the alcohol is, however, not nearly as powerful as that of the hydroxylamine. It should be noted that T.N.T. itself is a feeble methaemoglobin-former *in vitro* (cf. Voegtlin *et al.* 1920). We found that a saturated aqueous solution of T.N.T. added to an oxyhaemoglobin solution at 37° showed traces of methaemoglobin only after 2 hr., whilst under the same conditions the hydroxylamine produced methaemoglobin immediately, and the trinitrobenzyl alcohol within half an hour. The 4- and 6-aminodinitrotoluenes gave only traces of methaemoglobin after 4 hr. Voegtlin *et al.* (1920) showed that whilst injection of 2:6-dinitro-4-aminotoluene or tetranitro-4:4′-azoxytoluene caused no cyanosis in dogs, injection of dinitro-4-hydroxylaminotoluene, T.N.T., or 6-nitro-2:4-diaminotoluene caused a cyanosis which was particularly intense with the hydroxylamine. In our own experiments we noted cyanosis in rats within 5–10 min. after the injection of the hydroxylamine, within 10 min. after α-T.N.T. and within 30 min. to 1 hr. after β- and γ-T.N.T., all at a dose level of 500 mg./kg. in olive oil.

It is of value to endeavour to calculate the amounts of the different compounds isolated in terms of the T.N.T. administered. The yields of

dinitroaminotoluenes obtained from fractions A–C represent 6·1 % of the T.N.T. given. A less certain figure is available for the hydroxylamino compounds. Assuming that these were quantitatively converted to amino compounds during the acid hydrolysis used for estimation of the total amino compounds, the maximum amount which may be present represents 10 % of the T.N.T. administered; the admittedly incomplete yield of azoxy compound isolated, which was derived from originally existing hydroxylamino compound, represented 2·8 % of the T.N.T.; the best yield of hydroxylamino compound isolated as the *iso*benzaldoxime corresponded to 1 % of the T.N.T. given. On these figures, it may be reasonable to assess the hydroxylamino compounds as representing from 5 to 10 % of the T.N.T. administered. With the 6·1 % of dinitroaminotoluenes isolated, we can account for something like 15 % of the T.N.T. As to the form in which the remaining 85 % appears, the only established facts are, first, that 47 %, or rather more than one-half, appears as glucuronides, and secondly, that in addition to the 6·1 % of free dinitroaminotoluenes, 24 % of the T.N.T. appears as aromatic amino compounds with a free diazotizable amino group. If the latter were unconjugated, 71 of the 85 % would be accounted for. On the other hand, if, as we suggest, this 24 % is present as dinitroaminobenzylglucuronide, 37 % of the T.N.T. remains to be accounted for. Nor does the red pigment appear to account for any significant amount of the T.N.T., for our preliminary work on its isolation suggests that it represents only a very small proportion of the T.N.T. metabolites. As indicated in the introduction, there are many possible compounds to which T.N.T. may give rise, and further discussion at this stage is not profitable. As the next step we are therefore attempting to isolate and identify the conjugated compounds.

EXPERIMENTAL

Preparation of compounds

All α-T.N.T. used in this work had m.p. 81° and was prepared by recrystallization of good grade flaked T.N.T. from ethanol. β-T.N.T., m.p. 110–112°, and γ-T.N.T., m.p. 105°, were presented to us by Prof. C. R. Harington. 2:6-Dinitro-4-hydroxylaminotoluene, m.p. 141°, was prepared by the reduction of T.N.T. by H_2S in alcoholic ammonia (Anschütz & Zimmermann, 1915). 6-Nitro-2:4-diaminotoluene, m.p. 124°, was prepared according to Brady, Day & Reynolds (1929) and 2:4:6-trinitrobenzyl alcohol, m.p. 100°, according to Ganguly (1925).

2:4:6-Trinitro-*m*-cresol has been prepared by Datta & Varma (1919) by treating sulphonated *m*-cresol with nitrous fumes. We found that it could be prepared easily and in excellent yield by nitrating *m*-cresol dissolved in conc. H_2SO_4 according to the method described for preparation of picric acid from phenol (Cohen, 1924). It formed pale yellow needles, m.p. 109–110°, which became deep yellow on exposure to light. 2:6-Dinitro-4-amino-*m*-cresol, m.p. 159°, was prepared according to Kellner & Beilstein (1863).

2:4:6-Trinitrobenzoic acid, m.p. 210°, was prepared according to Conant (1922) and this acid was converted to 1:3:5-trinitrobenzene, m.p. 122°, by boiling with water (Conant, 1922).

2:2':6:6'-Tetranitro-4:4'-azoxytoluene. This compound is usually prepared from 2:6-dinitro-4-hydroxylaminotoluene, which is converted into a mixture of the azoxy compound and 2:6-dinitro-4-aminotoluene by heating with conc. HCl (Brand & Eisenmenger, 1913). We have prepared it from the hydroxylamine by oxidation with $K_2Cr_2O_7$ in H_2SO_4. 1 g. of the hydroxylamine was dissolved with mechanical stirring in 50 ml. 50 % (by vol.) H_2SO_4. Then 1 g. $K_2Cr_2O_7$ in 50 ml. water was added. The solution turned green and a pale yellow precipitate separated in good yield. The azoxy compound was filtered off, washed with water, dried and recrystallized from benzene (m.p. 215°). It may also be conveniently obtained from the more accessible 2:6-dinitro-4-aminotoluene. 12 g. ammonium persulphate were gradually added with mechanical stirring to 9·5 ml. ice-cold conc. H_2SO_4. The mixture was poured on to 70 g. crushed ice and 2·5 g. of the amino compound stirred into the mixture. The whole was allowed to stand 24 hr. at room temperature. The crude azoxy compound (2·3 g., m.p. 196–202°) was filtered off and dried. After recrystallization from toluene, it had m.p. 213–214°. The purest samples of the azoxy compound had m.p. 215–216°.

Animals used and preparation of the extracts

Rabbits were used in this work and experiments showed that in order to avoid losses it was desirable to limit the dose of T.N.T. to 150 mg./kg. on every fourth day. Twelve rabbits (2–3 kg.) were each given 0·3 g. finely powdered recrystallized α-T.N.T., suspended in water, by stomach tube. The urine was collected during the following 24 hr. from these and also from control rabbits receiving the same diet. Little material could be extracted from the urine as such or when it was made alkaline with Na_2CO_3. The urine was, therefore, acidified with 1/10 vol. of 10 % (by vol.) H_2SO_4 and extracted continuously with ether for 8 hr. The extract was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was a brownish tar (T_1). The urine collected during the second day was similarly treated (T_2). The combined extracted urines were now boiled for an hour and, after filtering and cooling, were extracted with ether. The acid-hydrolyzed extract was a dark brown tar (T_3).

The investigations described here relate only to the urine secreted in the 24 hr. following T.N.T. administration.

The above procedure was repeated until 45·9 g. of T.N.T. had been fed and the resulting urine extracted. The material obtained was weighed: T_1, 29·98 g.; T_2, 17·24 g.; T_3, 28·66 g. Control extractions showed that 70 % of the weight of T_1 could be obtained from normal urine. Very little of T_2 could be regarded as T.N.T. products. 26·34 g. of T_1 were dissolved in 450 ml. acetone and the solution was pipetted into 10 vol. of water. The resulting turbid solution was acid and was made alkaline by the cautious addition of Na_2CO_3. The solution was then extracted with ether until the mother liquor gave only a faint diazo reaction. Evaporation of the ether left a reddish-brown tar weighing 7·6 g.

Chromatographic analysis of T_1. The purified fraction T_1 (7·588 g.) was now dissolved in 500 ml. dry benzene and the solution poured into an alumina (Savory & Moore, Ltd., standardized according to Brockmann) column (1·9 cm. radius and 36 cm. long). The column was eluted with successive 100 ml. portions of dry benzene. Benzene eluted 5·306 g. of material from the column, whilst the total recovered by the use of ethanol, ether and alcoholic HCl to follow the benzene was 7·499 g. The eluates were combined into fractions according to the colours they gave in the diazo and Cumming reactions; details of these fractions are given in Table 6, fraction E being the material which could be eluted from the column only when ethanol, ether and alcoholic HCl were used.

Table 6. *Fractions of the* T.N.T. *extracts obtained by chromatography*

Fraction	Eluates from the column	Wt. (g.)	Appearance	Cumming reaction	Diazo reaction	Remarks
A	1– 5	1·804	Orange wax	Purplish blue	—	Mainly azoxy compounds
B	6–12	2·011	Orange powder	—	Red	Mainly amino compounds
C	13–32	1·257	Orange wax	Weak purple	Pale red	Amino compounds and little hydroxylamine
D	33–38	0·234	Reddish brown tar	Brownish purple	Pink	Hydroxylamine?
E	39–65	2·143	Dark brown tar	—	—	—

Isolation of 2:2′:6:6′-tetranitro-4:4′-azoxytoluene. Fraction A (1·8 g.) was dissolved in 34 ml. hot toluene. On cooling, 0·796 g. of the crude azoxy compound separated. This was purified by recrystallization from a benzene/ethanol mixture and 0·471 g. of pure 2:2′:6:6′-tetranitro-4:4′-azoxytoluene (pale yellow needles), m.p. and mixed m.p. 215°, was obtained. (Found: C, 41·7; H, 2·5; N, 21·3. Calc. for $C_{14}H_{10}O_9N_6$: C, 41·4; H, 2·5; N, 20·7%.) It gave a blue Cumming reaction, a negative diazo reaction and was identical in all respects with the synthetic material.

The second tetranitroazoxytoluene. The mother liquors from the separation of the above azoxy compound were evaporated, leaving a reddish tar (1·238 g.). The latter was dissolved in 20 ml. benzene and chromatographed on an alumina column without definite separation. The tar was dissolved in 10 ml. toluene, and on standing at 0° overnight 0·37 g. of crystalline material, of indefinite m.p., separated. This material was recrystallized first from toluene and then from benzene/ethanol (2 : 5), when a pale yellow crystalline product containing orange crystals was obtained. The orange crystals were separated by hand and had m.p. 165–167°; like the 4-azoxy compound they gave a negative diazo reaction and an intense purplish blue Cumming reaction and were sparingly soluble in most solvents.

Demonstration of the conversion of the hydroxylamine to the azoxy compound in urine. 50·2 mg. of 2:6-dinitro-4-hydroxylaminotoluene were shaken with 500 ml. normal rabbit urine. The mixture was acidified with ½ vol. 10% H_2SO_4. 400 ml. of the mixture (containing 33·1 mg. hydroxylamine) were now extracted continuously for 7 hr. with ether. The urine before extraction gave a pink Cumming test typical of a very weak solution of the hydroxylamine. The ether extract was evaporated to yield a hard brownish resin (199·2 mg.), which was dissolved in 5 ml. acetone and the solution pipetted into 50 ml. water. The aqueous solution was made just alkaline with Na_2CO_3 and extracted with three portions (45, 20 and 20 ml.) of ether. The ethereal solution was evaporated to a brownish tar (16·2 mg.) which gave the blue-purple Cumming reaction typical of the 4:4′-azoxy compound, though possibly still containing some hydroxylamine.

Tests for 2:6-dinitro-4-hydroxylaminotoluene in fresh T.N.T. *urine.* 3 ml. of the *cyclo*hexanone-methylethyl ketone reagent and 0·5 ml. 10% KOH were added to 0·5 ml. acidified (H_2SO_4) urine. The mixture was vigorously shaken for ½ min. and when the ketone layer had separated its colour was noted. All tests carried out in this way showed the pink colour typical of a dilute dinitrohydroxylaminotoluene solution. In no case was the blue Cumming reaction of the azoxy compound obtained. With special precautions a much more intense test could be obtained with the residue left from the ether extract of acidified T.N.T. urine. Thus when fresh peroxide-free ether was used, the residue gave the deep brown-red Cumming reaction of the hydroxylamine, but ether containing peroxides, such as recovered ether, gave a residue in which the Cumming reaction was a dirty blue. These results are summarized below:

Test material	Cumming reaction	Comment
T.N.T. urine	Negative	—
Acidified T.N.T. urine	Pink	Weak hydroxylamine reaction
Extract by peroxide-free ether	Red-brown	Hydroxylamine reaction
Extract by ether containing peroxides	Dirty blue	Reaction for an azoxy compound

2:6-Dinitro-4-aminotoluene and its derivatives. The 4-amino compound was prepared by H_2S reduction of pure α-T.N.T. in ethanol containing ammonia. Samples for comparison were also provided by Drs Morton & McGookin (University of Liverpool) and by Prof. Cumming (Royal Technical College, Glasgow). All samples were recrystallized from hot water and formed orange prismatic needles, m.p. 174°. The acetyl derivative formed colourless needles from ethanol, m.p. 227° (Körner & Contardi (1917) give m.p. 223°).

2:6-Dinitro-4-benzamidotoluene. This *compound* was prepared by means of benzoyl chloride and pyridine in the usual manner. It formed colourless rhombic plates from ethanol, m.p. 263–264°. (Found: C, 56·1; H, 3·7; N, 14·35. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6; N, 14·0%.)

2:6-Dinitro-4-monobenzenesulphonamidotoluene. Dinitro-4-aminotoluene (0·2 g.) was treated with benzenesulphonyl chloride (0·2 ml.) and pyridine (0·2 ml.), and the temperature raised to boiling for 1 min. The *product* was triturated with water and then dried and recrystallized from benzene. It formed thick colourless rectangular plates, m.p. 175–177°, turning a faint yellowish brown on prolonged exposure to light.

The dinitroaminotoluene, m.p. 135°. Electrolytic reduction of T.N.T. was carried out as described by Brand & Eisenmenger (1913). The dinitroaminotoluene was isolated from the product by extracting the brownish powder with large volumes of boiling water. It was recrystallized first from 10% acetic acid and finally from a large volume of water. The filtrates from the recrystallizations sometimes yielded red nitrodiaminotoluene, but this was not further investigated. The dinitroaminotoluene obtained in this way formed a deep yellow crystalline powder (needles), m.p. 130–135°, usually 133–134°. Recrystallization from benzene raised the m.p. to 135–140° and increased the size of the crystals, but had no particular advantage over water. (Found: C, 42·8; H, 3·7; N, 21·9. Calc. for $C_7H_7N_3O_4$: C, 42·65; H, 3·6; N, 21·3%.)

Dinitrobenzamidotoluene. The foregoing compound was benzoylated with benzoyl chloride and pyridine without external heating. The *product* was poured into water and then recrystallized from ethanol. It formed colourless needles, m.p. 220–230°, shrinking at 200°. (Found: C, 55·95; H, 3·8; N, 13·9. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6; N, 14·0%.)

Fractionation of the above benzoyl derivative. 2·2 g. of the benzoyl derivative were boiled up with three successive portions of 50, 50 and 75 ml. of absolute ethanol until completely dissolved. The three solutions on cooling deposited crystals (*a*) 0·634 g., m.p. 220–235°; (*b*) 0·475 g., m.p. 210–220°; (*c*) 0·373 g., m.p. 214–215° (sharply). The crystals (*c*) were recrystallized from ethanol and formed fine needles, m.p. 216–217°, and were identical with 2:4-*dinitro*-6-*benzamidotoluene* (see below). (Found: C, 55·6; H, 3·7; N, 13·8. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6; N, 14·0%.) Further fractionation of fractions (*a*) and (*b*) from ethanol, acetone and benzene gave a small amount, 50 mg., of 2:6-dinitro-4-benzamidotoluene as plates, m.p. 259–261°, from ethanol undepressed by the authentic 4-benzamido derivative.

Separation of the dinitroaminotoluenes as benzenesulphonyl derivatives. 0·5 g. of dinitroaminotoluene (m.p. 133°) (1 mol.) was dissolved in 0·5 ml. pyridine and 0·5 ml. benzenesulphonyl chloride (1·5 mol.). The mixture, which became hot, was heated to boiling for a moment, and allowed to cool; the semicrystalline mass was then triturated with water until it was completely solid. The solid was collected, dried and then boiled with 50 ml. of 95% ethanol and filtered. The residue (0·395 g., m.p. 216°) was recrystallized from benzene/ligroin, from which it separated in minute needles, m.p. 222°. The substance was 2:4-*dinitro*-6-*dibenzenesulphonamidotoluene*. (Found: C, 47·7; H, 3·0; N, 8·7; S, 13·2. Calc. for $C_{19}H_{13}O_8N_3S_2$: C, 47·8; H, 3·2; N, 8·8; S, 13·4%.) After separation of this compound, the filtrate was cooled when a small crystalline fraction (38 mg.), melting indefinitely at 228–238°, was removed. The alcoholic filtrate was now diluted with 3 vol. water and allowed to stand overnight. The crystalline precipitate which had formed was filtered off and dried (m.p. 170°; yield 0·336 g.). The 2:6-*dinitro*-4-mono*benzenesulphonamidotoluene* was now recrystallized from benzene/ligroin when it formed thick rectangular plates, m.p. 177–178°. (Found: C, 46·7; H, 3·4; N, 12·5; S, 9·6. Calc. for $C_{13}H_{11}O_6N_3S$: C, 46·3; H, 3·3; N, 12·5; S, 9·5%.).

Preparation of the dinitroaminotoluenes from the pure benzenesulphonyl compounds

2:4-*Dinitro*-6-*aminotoluene*. The foregoing 2:4-dinitro-6-*di*benzenesulphonamidotoluene (1·16 g.) was dissolved with heating and stirring in 10 ml. of 80% H_2SO_4. When it had dissolved, the solution was poured on ice and the yellow precipitate filtered and dried (yield 0·435 g. = 90%). The 2:4-*dinitro*-6-*aminotoluene* was recrystallized from water; it formed bright yellow needles, m.p. 176°. (Found: C, 43·0; H, 3·7; N, 21·4. Calc. for $C_7H_7N_3O_4$: C, 42·65; H, 3·6; N, 21·3%.) On admixture with 2:6-dinitro-4-aminotoluene (m.p. 174°), the m.p. was depressed to 135–140°. On treatment with benzenesulphonyl chloride and pyridine, the dibenzenesulphonyl derivative was regenerated (m.p. and mixed m.p. 222°).

2:4-*Dinitro*-6-*acetamidotoluene*. 150 mg. 2:4-dinitro-6-aminotoluene were dissolved in 0·5 ml. pyridine and acetyl chloride was added dropwise until the yellow colour disappeared. The *product* was triturated with water, dried and recrystallized from benzene. It formed faintly yellow needles, m.p. 159–160°. (Found: C, 45·6; H, 3·8; N, 17·7. Calc. for $C_9H_9N_3O_5$: C, 45·2; H, 3·8; N, 17·6%.)

Benzoylation of the above 6-amino compound gave 2:4-*dinitro*-6-*benzamidotoluene* (m.p. 215–216°) (found: C, 56·0; H, 3·8. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6%), identical with the benzoyl compound obtained by fractionation of the benzoyl derivatives of the dinitroaminotoluene, m.p. 135° (see above).

2:6-*Dinitro*-4-*aminotoluene*. The monobenzenesulphonyl compound (0·3 g.) was hydrolyzed by dissolving in 5 ml. hot 80% H_2SO_4. The solution was poured on ice and the orange precipitate filtered off and dried (m.p. 168°; yield 80%). On recrystallization from water, it formed orange-yellow needles, m.p. 174° undepressed by authentic 2:6-dinitro-4-aminotoluene but considerably depressed by 2:4-dinitro-6-aminotoluene.

Examination of fraction B (Table 6)

The fraction (2·011 g.) was boiled with six 100 ml. portions of water and filtered. When they were cooled the first four portions deposited 1·032 g. of a yellow crystalline powder (B_1), m.p. 134°, analyzing correctly for a dinitroaminotoluene. (Found: C, 42·9; H, 3·6; N, 22·0. Calc. for $C_7H_7N_3O_4$: C, 42·65; H, 3·6; N, 21·3%.) A small quantity was acetylated with pyridine and acetyl chloride and the product purified by recrystallization from aqueous ethanol. The acetyl derivative obtained formed needles, m.p. 226°, which did not depress the m.p. of authentic 2:6-dinitro-4-acetamidotoluene. B_1 therefore contained 2:6-dinitro-4-aminotoluene. On benzoylation, B_1 gave needles of a benzoyl derivative melting indefinitely between 220–230° after softening at 200°.

Separation of 2:4-*dinitro*-6-*aminotoluene and* 2:6-*dinitro*-4-*aminotoluene from fraction* B_1. 0·7 g. B_1 was dissolved in 0·8 ml. benzenesulphonyl chloride and 1 ml. pyridine and the solution raised just to boiling. The product was allowed to cool and was then triturated with water until it solidified and broke up (yield 1·39 g.). The dried mixture of benzenesulphonyl derivatives was now boiled with 50 ml. absolute ethanol and filtered. The white residue (0·495 g., m.p. 215–216°) was recrystallized from benzene/ligroin and was identical with 2:4-dinitro-6-dibenzenesulphonamidotoluene. It formed minute needles, m.p. 221–222° and mixed m.p. 222°. (Found: N, 9·1; S, 13·9. Calc. for $C_{19}H_{13}O_8N_3S_2$: N, 8·8; S, 13·4%.) The substance (130 mg.) was hydrolyzed with 80% H_2SO_4 and the product poured on ice. The yellow crystalline precipitate (50 mg.) was filtered off, washed with water and dried; it had m.p. 175–176° undepressed by authentic 2:4-dinitro-6-aminotoluene. The

mixed m.p. with 2:6-dinitro-4-aminotoluene (m.p. 174°) was 134–143°.

The alcoholic filtrate from the dibenzenesulphonyl derivative was cooled, and a small crystalline fraction (66 mg., m.p. ndefinite 220–235°) was filtered off and discarded. Three volumes of water were now added to the alcoholic filtrate and the whole was allowed to crystallize overnight. The flocculent, crystalline precipitate was collected and dried. In the crude state it had m.p. 146–155° (yield 0·714 g.). On recrystallization from benzene/ligroin it formed thick rectangular plates, identical with 2:6-dinitro-4-monobenzenesulphonamidotoluene; its m.p. was 175–177° and the mixed m.p. 176–177°. (Found: N, 12·3; S, 9·7. Calc. for $C_{13}H_{11}O_6N_3S$: N, 12·5; S, 9·5%.) On hydrolysis with 80% H_2SO_4 and recrystallization from water followed by benzene, it gave the parent 2:6-dinitro-4-aminotoluene as orange needles, m.p. 174° undepressed by the authentic 4-amino compound but depressed to 135–145° by the 6-amino compound.

Fraction B_2. The fifth and sixth water extracts of B gave a less pure crystalline material (0·393 g.). This deposit was combined with the product obtained on evaporation of the mother liquors from B_1, and the whole was recrystallized from boiling water. The yellow crystalline product, B_2 (0·411 g.) melted at about 140° after shrinking at 131°. Its colour reactions showed it to contain only dinitroaminotoluene; it was very similar to B_1, and was presumably a mixture of the 4- and 6-aminodinitrotoluenes.

Fraction B_3. By evaporation of and crystallization from 50 ml. water, there was obtained from the mother liquors of B_2 a further fraction, B_3 (69 mg.), m.p. 129°, almost identical in properties with fraction B_1.

Examination of fraction C (Table 6)

The fraction (1·26 g.) was boiled with two successive portions of water. The first portion deposited on cooling a yellow crystalline powder, C_1 (0·265 g.), m.p. 132–134°, identical with fraction B_1. The second portion deposited fraction C_2 (0·151 g.), a yellow crystalline powder, m.p. 128°. C_2, however, contained traces of a dinitrohydroxylaminotoluene, since, although like the previous fractions it gave an intense diazo reaction, it differed from them in giving a weakly positive brown Cumming reaction, and it was faintly reducing to Benedict's reagent. Attempts to isolate the hydroxylamine failed owing to lack of material. The residue, C_3, was a brownish-red glass from which nothing could be crystallized.

Examination of fraction D (Table 6)

The fraction was a brownish tar (0·265 g.) and its colour reactions indicated the presence of the hydroxylamine. The diazo reaction was faint red; it was faintly reducing to Benedict's reagent and it gave a brown-red Cumming reaction. Attempts to prepare the crystalline *iso*benzaldozime failed.

N-(3:5-dinitro-4-methyl phenyl)-isobenzaldoxime

0·295 g. (1 mol.) of 2:6-dinitro-4-hydroxylaminotoluene and 0·2 g. (1½ mol.) benzaldehyde were gently heated together until they melted completely. The product crystallized on cooling and was recrystallized from benzene. The pure *isoaldoxime* (0·226 g.) formed pale yellow needles, m.p. 213°. (Found: C, 55·8; H, 3·7; N, 14·8. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6; N, 14·0%.) When 2:6-dinitro-4-aminotoluene is heated with benzaldehyde no condensation takes place and the amine can be recovered unchanged.

Isolation of 2:6-dinitro-4-hydroxylaminotoluene from T.N.T. urine

Twelve rabbits on a diet of cabbage and swedes were each fed 0·5 g. T.N.T. with water. The urine (1460 ml.) was collected during 24 hr., acidified with $\frac{1}{10}$ vol. of 10% H_2SO_4, filtered through cotton-wool and then extracted continuously with peroxide-free ether for 3 hr. This extract was now worked up, the residual urine being extracted with fresh ether for a further 3 hr. Both extracts were worked up in the same way in that they were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, leaving an orange-brown resin. The resin was dissolved with gentle warming in 0·5 ml. benzaldehyde and when homogeneous the mixture was heated to boiling for 1 min. On cooling to 0° a semi-crystalline mass was produced. This was taken up in 5–10 ml. boiling benzene, charcoaled and filtered. The filtrate was kept overnight uncovered at room temperature. The dish now contained a dark orange semi-crystalline mass. This was triturated with absolute ethanol which dissolved tar and benzoic acid, leaving pale yellow needles which were collected and dried. The yield of crude *iso*aldoxime was 68 mg. from the first extraction and 44 mg. from the second. Wherever small or zero yields were encountered, tests on the resinous product always indicated that considerable amounts of the azoxy compound had been formed.

135 mg. of the crude *iso*aldoxime were dissolved in 5 ml. hot benzene, the solution being charcoaled and filtered. On cooling, 39·3 mg. of very pale yellow needles, m.p. 180–190°, separated. The filtrate was treated with 3 vol. ligroin, which threw out 11·6 mg. of yellow needles; these gave an immediate red-brown Cumming reaction and reduced ammoniacal $AgNO_3$ and Benedict's reagent. The second compound had m.p. 130–134° and appeared to be free 2:6-dinitro-4-hydroxylaminotoluene. Further recrystallization of the *iso*aldoxime from benzene gave a product, m.p. 211–213°, identical with *N*-(3:5-dinitro-4-methylphenyl)-*iso*benzaldoxime (m.p. 212–213°); the mixed m.p. was 211–212°. The *iso*aldoxime gave a Cumming reaction which developed slowly, being colourless at first, then becoming pink and finally brown-red. It did not reduce Benedict's reagent or ammoniacal $AgNO_3$ and did not give a colour in the diazo reaction. (Found: C, 55·9; H, 3·7; N, 13·6. Calc. for $C_{14}H_{11}O_5N_3$: C, 55·8; H, 3·6; N, 14·0%.)

The yellow hydroxylamine obtained from the mother liquors of the crude *iso*aldoxime—none could be obtained from the mother liquors of the purified product—was recrystallized from benzene/ligroin. The yellow needles obtained had m.p. 139°. It did not depress the m.p. of authentic 2:6-dinitro-4-hydroxylaminotoluene and was identical with it in all respects.

Estimation of diazotizable reduction products in T.N.T. urine

Reagents used: 2N-HCl; 0·1% $NaNO_2$; 0·5% ammonium sulphamate; 0·1% aqueous *N*-(1-naphthyl)-ethylenediamine hydrochloride; a solution of 2:6-dinitro-4-aminotoluene in N-HCl containing 0·2 mg. base/ml.

Standard curve. 4 ml. 2N-HCl are added to 2 ml. of the solution of dinitroaminotoluene in normal rabbit urine, followed in turn, with mixing, by 2 ml. $NaNO_2$, 2 ml.

ammonium sulphamate and 2 ml. of the diamine hydrochloride solution. The mixture is then made up to 25 ml. with absolute ethanol. It is mixed and allowed to stand 1 hr., then read in the Spekker photoelectric absorptiometer, using an Ilford no. 602 blue filter. Drum readings are then plotted against the number of mg. of 4-amino derivative in 2 ml. solution (see Fig. 1).

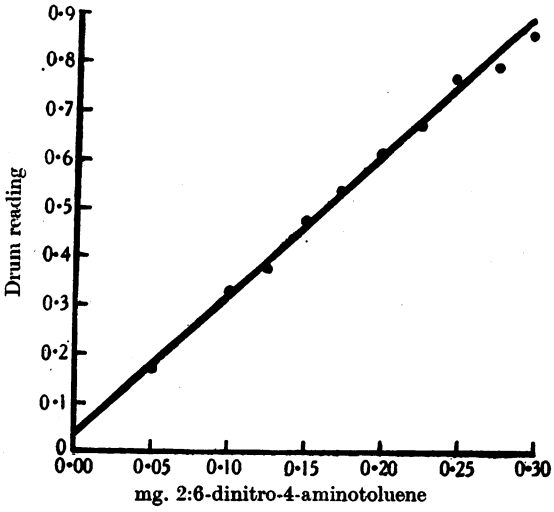


Fig. 1. Calibration curve for the estimation of 2:6-dinitro-4-aminotoluene in rabbit urine by the Spekker photoelectric absorptiometer.

Estimation in urine. The above procedure was repeated with filtered urine diluted to 5 vol. with water instead of the standard dinitro base solution. That 2:6-dinitro-4-aminotoluene added to T.N.T. rabbit urine could be recovered almost quantitatively is shown by the following examples:

Calc. (mg./ml.)	Found (mg./ml.)
0·2	0·195
0·1	0·098

Two groups of rabbits were used. The first group was fed daily on 100 g. carrots, 100 g. swedes and 100 g. cabbage, and on this 'wet diet' each rabbit excreted an average of 200 ml. urine/day. The second group was given a 'dry diet' of 50 g. bran and 100 g. cabbage, and the urine volume of each rabbit was about 50 ml./day. Control urine was collected on the first day, and blank determinations were carried out with this. The blank value corresponded to 0–2 mg./day of the 4-amino compound, the blank being greatest with the 'dry diet'. On the morning of the second day T.N.T. was fed by stomach tube. The following two examples illustrate the type of result obtained:

T.N.T. fed (mg.)	Diet	Amino compound excreted (mg./day) during day 1	2*	3	4
170	Wet	0	36·2	1·0	0
750	Dry	2·8	131·0	24·6	4·7

* T.N.T. fed on day 2.

For the estimations on hydrolyzed urine, 10 ml. urine and 10 ml. 2N-HCl were boiled for 20 min. and the solution made up to 25 ml. The results are given in Table 3.

Glucuronic acid excretion

Glucuronic acid was estimated by a method in which the intensity of colour of an amyl alcohol extract of the pigment formed from glucuronic acid and naphthoresorcinol was measured by means of the photoelectric absorptiometer. A description of this method will be published later, as it is of general application to the estimation of glucuronic acid in urine.

Rabbits were divided into two groups, fed on the diets described above. Each experiment was carried out over 5 or 6 days, T.N.T. being administered orally on the third day.

Experience showed that on doses of 250 mg./kg. or less of T.N.T. the glucuronic acid was excreted within 24 hr. Since there is a daily variation in the excretion of glucuronic acid by the rabbit even on a constant diet, the mean value of the excretion of the first, second, fourth and fifth day was subtracted from that of the third day in order to obtain the increase due to the T.N.T. administered. For doses of T.N.T. above 250 mg. the excretion takes 48 hr., and, therefore, from the excretion on the third and fourth days, there was subtracted the mean value of that on the first, second, fifth and sixth days. In all cases, the T.N.T. was given at the beginning of the third day.

Ethereal sulphate excretion

Ethereal sulphate excretion was determined as described by Williams (1938).

The red pigment. Preparation of injected compounds

2:4:6-*Trinitrobenzyl acetate.* 200 mg. of the alcohol were treated with 15 drops of acetyl chloride and the mixture was heated at 70–80° for 10 min. On cooling, the liquid mixture crystallized completely (yield almost theoretical). The solid was triturated with water and recrystallized from ethanol. The *acetate* formed colourless plates, m.p. 73°. (Found: C, 37·8; H, 2·3; N, 14·9. Calc. for $C_9H_7N_3O_8$: C, 37·9; H, 2·5; N, 14·7%.)

2:4:6-*Trinitrobenzylmethyl ether.* 2:4:6-Trinitrobenzyl bromide was dissolved in dry methanol and the calculated amount of sodium metal added. The NaBr was filtered off and the brown solution evaporated. The tarry residue was dissolved in benzene and the ether was thrown out with light petroleum. After several repetitions of the latter process a small amount of the ether was obtained as brown powder, m.p. 157°. No solvent for its recrystallization was found, so that purification for analysis was not possible.

Trinitromesitylene, m.p. 238°, was prepared according to Fittig (1867).

SUMMARY

1. From the urine of rabbits receiving 2:4:6-trinitrotoluene (α-T.N.T.), there have been isolated 2:6-dinitro-4-hydroxylaminotoluene, 2:6-dinitro-4-aminotoluene and 2:4-dinitro-6-aminotoluene, showing the existence of the reduction mechanism

$$CH_3.C_6H_2.(NO_2)_3 \rightarrow CH_3.C_6H_2.(NO_2)_2.NHOH \rightarrow CH_3.C_6H_2.(NO_2)_2.NH_2.$$

2. The 2:2′:6:6′-tetranitro-4:4′-azoxytoluene isolated by previous workers is not a metabolic product; it is absent from freshly voided urine and is formed from dinitrohydroxylaminotoluene during the extraction procedures.

3. Information was sought on the nature of the red pigment present in T.N.T. urine, by administering possible intermediate substances. Of 15 substances used, including α-, β-, γ-T.N.T. and the metabolic products previously isolated, only α-T.N.T. and 2:4:6-trinitrobenzyl alcohol caused red-pigment excretion; the acetate, but not the methyl ether of trinitrobenzyl alcohol, also caused red-pigment formation. The alcohol may thus be a constituent part or a precursor of the red pigment, and this finding demonstrates the probable existence of the oxidation mechanism

$$CH_3.C_6H_2.(NO_2)_3 \rightarrow CH_2OH.C_6H_2.(NO_2)_3.$$

4. 47% of the T.N.T. administered is excreted as glucuronides and 30% as aromatic amino compounds estimated as 2:6-dinitro-4-aminotoluene. The aromatic amino compounds include not only dinitroaminotoluenes but possibly dinitroaminobenzyl alcohol conjugated with glucuronic acid. Trinitrobenzylglucuronide is also a likely constituent.

5. Doses of T.N.T. up to 150 mg./kg. are eliminated in 24 hr.: larger doses require up to 48 hr. The volume of urine secreted by rabbits receiving T.N.T. was varied widely by dietary means, without effect on the rate of excretion of the administered T.N.T. or its toxicity.

6. The delicate colour reactions given by α, β- and γ-T.N.T., and a considerable number of possible T.N.T. metabolic products and their derivatives, when treated with potassium hydroxide and a mixture of methylethylketone and *cyclo*hexanone, are described.

7. The significance of the chemical findings is discussed in relation to the toxicity of T.N.T.

8. 2:4-Dinitro-6-aminotoluene and a number of other compounds related to T.N.T. have been prepared for the first time.

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