Enzymic Reduction of 2:4-Dinitrophenol by Rat-tissue Homogenates

By V. H. PARKER

*Medical Research Council Unit for Research in Toxicology, Serum Research Institute,
Carshalton, Surrey*

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During an investigation into the toxic properties of 3:5-dinitro-*o*-cresol (DNOC) and 2:4-dinitrophenol (DNP) the breakdown of these substances by animal tissues *in vitro* was studied. Dinitrophenol and dinitro-*o*-cresol have been studied as inhibitors of phosphorylation in various enzyme systems and have been shown to affect the metabolism of tissues *in vitro*, but little has been reported of the effect of such systems on the dinitrophenol molecule. There is some information on the metabolic products of dinitrophenol by Magne, Mayer & Plantefol (1932) who state that the excretion products in the urine after administration of dinitrophenol to man and dogs were unchanged dinitrophenol and 2-amino-4-nitrophenol. Later, Greville & Stern (1935) demonstrated that 2-amino-4-nitrophenol was the main product after dinitrophenol had been in contact with a bacterial succinic dehydrogenase system.

The present studies were undertaken in order to ascertain the possible metabolic products of DNP and DNOC, and the organs mainly responsible for the metabolic change. As three of the amino derivatives of dinitrophenol were readily obtained, most of the following experiments were done with this compound in preference to DNOC.

EXPERIMENTAL

Preparations. Rats were killed by a blow on the head and immediately afterwards their throats were cut and the bodies bled out.

Liver homogenate. The tissue was removed, weighed and homogenized in 15 ml. of 0.06M-phosphate buffer, pH 7.4, for 1 min. Phosphate buffer was then added to make the final proportions of 3 parts of buffer to 1 part of tissue. Unless otherwise stated the homogenate was used at once.

Liver extracts. These were made by grinding a known weight of liver with washed sand and phosphate buffer in a pestle and mortar. After centrifugation, the supernatant was removed and used in volumes equivalent to 2.5 g. of homogenized liver.

Liver brei. This was prepared by mincing liver in a domestic mincing machine.

Acetone-dried liver. This was prepared by homogenizing 25 g. liver in 100 ml. of ice-cold acetone. The mixture was centrifuged and the supernatant discarded. The residue was resuspended in 100 ml. cold acetone and recentrifuged. The solid residue was washed with 100 ml. cold ether. After

centrifuging, the solid residue was spread to dry on filter paper. The dried powder was used at once.

Pieces of whole liver (approximately 0.5 g.) were freeze-dried in small bottles in a centrifugal freeze-dryer.

Homogenate experiments. For the study of the enzymic process, 8 ml. of homogenate were measured into a test tube. Inhibitors or activators also prepared in buffer solution were added at this stage. To this was added 4 ml. of dinitrophenol 0.5 mg./ml. prepared in buffer solution, pH 7.4. Finally, the volume was made up to 20 ml. with buffer solution giving a final concentration of dinitrophenol of 5.4×10^{-4} M. The contents of the tube were mixed by shaking and the tube placed in a water bath at 37°. A control tube was prepared each time containing the same concentration of dinitrophenol and inhibitor or activator in a total volume of 20 ml. of buffer solution. At intervals of 0.5 hr., 0.5 ml. samples were removed with a Krogh-Keys syringe pipette and analysed for dinitrophenol or aminonitrophenol according to the requirements of the experiment. The buffer solution, pH 7.4, was 0.06M-phosphate containing 7×10^{-2} M-succinate.

Determination of DNP. Dinitrophenol was estimated by the method based on extraction of the alkali salt with methyl ethyl ketone followed by colorimetry (Parker, 1949).

Determination of aminonitrophenols. The proteins of 0.5 ml. homogenate were precipitated by the addition of 1 ml. 15% (w/v) trichloroacetic acid. The mixture was centrifuged, 1 ml. supernatant was mixed with 2 ml. water and 1 ml. 5N-HCl. The solution was then cooled in ice water and placed in a dark cabinet. This was necessary because it was found that the diazonium compounds in solution were decomposed by daylight. Diazotization was carried out by the addition of 0.5 ml. 0.2% NaNO₂ solution, mixing and leaving in the dark for 10 min. Excess HNO₂ was removed by addition of 1 ml. 1% ammonium sulphamate solution. After a further 10 min. the diazonium compound was coupled with 2 ml. 0.5% *N*-1-naphthylethylenediamine prepared in 3N-HCl. The tube was placed in a water bath at 37° and the colour developed for 30 min. At the end of this time, the optical density of the solution was measured at 550 mμ. on a Unicam D.G. spectrophotometer. Standard curves were prepared from the two isomeric aminonitrophenols.

Extraction of the ether-soluble amino compound from liver homogenates. Liver homogenate (50 g.) was prepared as above. DNP concentration used was 100 μg. DNP/ml. After incubation for 1 hr. at 37° the reaction was stopped and the protein precipitated by 15% trichloroacetic acid, cooled and centrifuged. The supernatant liquor was made just alkaline with NH₄OH and then just acid to litmus paper with acetic acid. The solution was then extracted three times with

redistilled ether. The combined ether extracts were dried and evaporated to dryness. The brownish residue was dissolved in a small amount of ethanol (1–2 ml.).

Chromatography. A chromatograph column was prepared in an all-glass apparatus using a sludge of ethanol and alumina (P. Spence and Sons Ltd. 778–780 Salisbury House, London, E.C. 2. Activated alumina, Type H). The ethanolic extract of liver homogenate was placed on the top of the column and the chromatogram developed with ethanol containing a little NH_3 (approximately 0.15 N). The rate of development was increased by the application of a small positive pressure to the top of the column. As a rule, three bands were seen moving separately down the column. The last band was eluted by changing the composition of the developing fluid to one of 90% ethanol containing 0.15 N- NH_3 . Each component was collected separately as it appeared.

Paper-partition chromatography. A pencil line was drawn across a strip of Whatman filter paper no. 3 (25 × 3.5 cm.) at a distance of 4 cm. from one end, a second pencil line at 15.0 cm. from the first. A small quantity (10–30 μl .) of the ethanolic extract of liver homogenate was placed on the lower line and dried. With each chromatogram of the unknown substance a control was run consisting of a mixture of DNP and the isomeric amino compounds. A spot of this mixture was placed on the line on one side of the 'unknown' spot. The strip was suspended in a gas jar so that the end of the paper nearest to the spots of solutions was just dipping into the developing fluid. The gas jar was filled with N_2 by displacement of air and closed with a greased lid and development of the chromatogram allowed to take place by upward capillary attraction until the liquid front reached the second pencil line. At this stage the paper was removed and allowed to dry in the air. The developing fluid consisted of 20% benzene, 20% pentanol, 40% ethanol and 20% ammonia solution (sp.gr. 0.880). All percentages were v/v.

Spectroscopy. Some of the solutions obtained by chromatography were examined for ultraviolet absorption in the Unicam Quartz S.P. 500 spectrophotometer. The absorption curves were determined from 250 to 400 $\mu\mu$. at a slit width of 0.1 mm.

All but two of the following experiments took place in buffered solutions at pH 7.4. The conditions for the two exceptions are mentioned in the text.

RESULTS

Characteristics of the enzyme preparation. The activity of the homogenates, measured as μg . DNP destroyed per ml. of solution in 1 hr. was at a maximum when the liver was prepared in phosphate buffers at pH values of 7.0–7.5.

The optimum temperature was approximately 37°. Heating the homogenate to 100° resulted in complete loss of activity, whilst maintaining the preparation at 60° for 10 min. resulted in a decrease in activity by 70%. There was no destruction of DNP when the homogenate was kept at 0°.

The activity of the preparation depended upon the length of time taken to homogenize the liver and upon the age of the homogenate. Preparations homogenized for periods of time varying from

30 sec. to 6 min. exhibited varying degrees of activity, the most active preparation being that which had been homogenized for 1 min. Experiments also showed that the homogenate was most active if used immediately after preparation. Left for periods of up to 2 hr. at 37° the homogenates lost 20% of their activity. From 2 to 4 hr. there was little further loss in activity.

Michaelis constant, K_m and effect of enzyme concentration. In order to determine the Michaelis constant for the reaction, it was necessary to remove samples from the homogenate at intervals of 5 min. The reciprocals of the amount of DNP destroyed in 5 min. ($1/v$) were plotted against the reciprocals of the concentration ($1/c$) as suggested by Lineweaver & Burk (1934). The Michaelis constant was derived from the slope of the line, K_m/v_m , and it was found to be 8.5×10^{-5} M-DNP (Fig. 1).

Fig. 1. Effect of DNP concentration within the range of 5.4×10^{-5} to 5.4×10^{-4} M on the amount of DNP destroyed by liver homogenates. v = moles DNP destroyed in 5 min.

Fig. 2 shows the result of varying the concentration of the homogenate. There is a linear relationship between the amount of enzyme present and the amount of DNP destroyed when not more than 80% of DNP is destroyed.

Comparison of homogenates with other preparations. When the activities of liver extract and liver brei were compared with the activity of an equivalent quantity of liver homogenate it was found that under identical conditions there was no difference between the three forms of preparations. In other experiments, acetone-dried liver and freeze-dried liver were compared with homogenates. Acetone-dried liver powder equivalent to 4 g. of fresh liver destroyed only 30% of DNP (initial concn. 5.4×10^{-4} M) in 1 hr. compared with 60% of DNP destroyed by 2.5 g. of homogenized liver in the same time. Whole liver freeze-dried had approximately

90% of the activity of homogenized liver. Freeze-dried liver retained its initial activity in a refrigerator for two weeks.

Fig. 2. Effect of enzyme concentration on amount of DNP destroyed. Enzyme concentration is expressed as ml. of liver homogenate in range of 2–9 ml. (15 g. liver homogenized and made up to a final volume of 60 ml.). Each solution analysed after 1 hr. at 37°. Initial DNP concentration 5.4×10^{-4} M.

An attempt was made to determine the active portion of the homogenate. An homogenate was divided into three portions A, B and C. A was incubated with DNP in the usual way. B was centrifuged

Table 1. Activity of liver homogenate fractions

(Preparation A, 2.5 g. liver homogenized, DNP added and volume adjusted to 20 ml. Preparation B, 2.5 g. liver homogenized in 10 ml. buffer solution, centrifuged, DNP added to supernatant fluid. Volume adjusted to 20 ml. Solid residue from B suspended in buffer solution, DNP added and volume adjusted to 20 ml. Preparation C, 2.5 g. liver homogenized in 10 ml. of buffer solution, centrifuged and supernatant fluid filtered through kieselguhr. Filtrate adjusted to 20 ml. after addition of DNP. DNP concentration, 100 µg./ml., pH 7.4.)

Preparation	Whole homogenate of A	Super-natant of B	Residue of B	Filtered super-natant of C
µg. DNP destroyed per ml. after 60 min.	56	41	13	8
Activity expressed as percentage activity of whole homogenate	100	73	23	15

at the top speed of an angle centrifuge and the supernatant fluid and solid residue incubated separately with DNP. C was also centrifuged, but the supernatant fluid was filtered through a layer of kieselguhr before assessment of its activity. Table 1 indicates that most of the activity resides in the

opalescent supernatant fluid after centrifuging, but is lost by filtration.

Effect of enzyme inhibitors. The following compounds were examined for their effect on the activity of liver homogenates: potassium cyanide, *p*-chloromercuribenzoic acid (CMBA), iodoacetic acid, sodium pyrophosphate, sodium azide and sodium malonate. Table 2 shows that only cyanide inhibited the destruction of dinitrophenol.

Table 2. Effect of inhibitors on destruction of DNP

(For each test, 2.5 g. of liver were homogenized in 15 ml. of phosphate buffer, and 1 ml. of inhibitor added. After incubation at 37° for 30 min., DNP was added to a final concentration of 5.4×10^{-4} M and a total volume of 20 ml. Samples were removed for analysis at 30 and 60 min.)

Compound	Final concn. (M)	Inhibition (%)
Potassium cyanide	5×10^{-5}	32
	5×10^{-4}	90
	5×10^{-3}	100
Sodium azide	5×10^{-3}	8
Iodoacetic acid	5×10^{-3}	8
<i>p</i> -Chloromercuribenzoic acid	5×10^{-3}	0
Sodium pyrophosphate	5×10^{-2}	0
Sodium malonate	5×10^{-3}	0

Other factors influencing the breakdown of DNP. Greville & Stern (1935) and Westfall (1943) used a dehydrogenase system to demonstrate the reduction of nitro groups in dinitrophenol and trinitrotoluene. The effect of substrates for dehydrogenases was observed on the activity of liver homogenates in destroying DNP. It was found that their activity could be increased by almost 20% when 7×10^{-2} M-succinate or lactate was added. The experiments reported above were carried out under aerobic conditions. Westfall demonstrated that the reduction of trinitrotoluene also occurred under anaerobic conditions. The rate of decomposition of DNP under aerobic and anaerobic conditions were compared. DNP was added to chilled liver homogenate in Thunberg tubes immersed in ice water. The DNP concentration was 5.4×10^{-4} M after adjustment to a known volume with phosphate buffer, pH 7.4. The tubes were evacuated and refilled with nitrogen three times in succession. These tubes as well as similar tubes, open to the atmosphere, containing the same amount of liver homogenate and DNP were incubated at 37° for 1 hr. It was found that the reaction took place at the same rate under aerobic and anaerobic conditions.

That the succinic oxidase system was not responsible for the reduction of dinitrophenol was demonstrated by the following experiment. Succinic oxidase was prepared by Slater's modification of Keilin & Hartree's method (Slater, 1949). The final preparation was such that 0.5 ml. of a 1 in 25 dilution

Table 3. *Enzymic activity of rat and rabbit tissues*

(Spleen, kidney, heart, intrascapular fat and brain tissues were removed from the freshly killed animal, weighed and homogenized in 3 parts of phosphate buffer. Liver and muscle, 2-3 g. weighed and homogenized in 3 parts of phosphate buffer. DNP added to a final concentration of 5.4×10^{-4} M. Blood, 5 ml. taken, DNP in phosphate added to a final concentration of 5.4×10^{-4} M-DNP. All homogenates incubated at 37° and analysed after 30 min. DNP destroyed calculated in terms of mg./100 g. wet wt. tissue in 30 min.)

	Rat tissue		Rabbit tissue	
	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)	DNP destroyed in 30 min. (mg./100 g. tissue)	Activity (%)
Liver	68	100	29	100
Spleen	40	59	0	0
Kidney	41	60	12	41
Heart	20	29	1	3
Intrascapular fat	33	47	—	—
Muscle	11	16	—	—
Brain	2	3	—	—
Blood	0	0	0	0

reduced 1 ml. 10^{-5} M-methylene blue in 30 min. For the determination of its effect on DNP 1 ml. of the undiluted enzyme preparation was used. The buffer solution, pH 7.7, was 0.01 M-phosphate solution containing 0.03 M-sodium bicarbonate. The Thunberg tubes were filled with 5% carbon dioxide in nitrogen after removal of air. It was found that with DNP concentrations of 4.5×10^{-5} to 4.5×10^{-4} M no destruction of the nitro compound occurred.

Distribution of the enzymic activity. The activity of other tissues of the rat was examined by making homogenates from them and incubating with dinitrophenol under identical conditions.

Table 3 shows that the activity of the rabbit tissue is lower than that of the corresponding rat tissue. On the other hand, when injected into the living animal, DNP disappears from the blood stream of the rabbit within a few hours, whereas it remains in the blood of the rat for several days (Parker, Barnes & Denz, 1951).

Reduction of DNOC. Experiments were performed with rat homogenates in which DNOC was substituted for DNP. It was found that these homogenates could reduce DNOC and the rate of reduction was measured as rate of disappearance of DNOC. Various tissues had similar relative capacities of reducing DNOC as they had in reducing DNP. The product of DNOC was a diazotizable amine, but was not identified.

The reduction product of DNP

The experiments described above indicated that DNP was destroyed by liver homogenates by an enzymic process. The products of this reaction were sought. A homogenate which had been in contact with DNP for 1 hr. was taken. The reaction was stopped by addition of 5 ml. of 20% trichloroacetic acid to every 20 ml. of homogenate. A portion of the supernatant was diazotized and coupled with *N*-1-naphthylethylenediamine. A magenta dye

was produced. On the assumption that only primary aromatic amines form dyes of this kind, it was decided that at least one of the metabolic products was such an amine. The possible amino derivatives of 2,4-dinitrophenol are 4-amino-2-nitrophenol, 2-amino-4-nitrophenol and 2,4-diaminophenol. The isomeric nitroamines can be separated from the diamine by simple ether extraction in acid solutions, and it was found that the greater part of the amino fraction could be extracted in this way.

Fig. 3. Absorption curves of diazo dyes of aminonitrophenols. Aminonitrophenol diazotized (25 μ g.) and coupled with *N*-1-naphthylethylenediamine in a final volume of 7.5 ml. —, dye of 2-amino-4-nitrophenol; - - -, dye of 4-amino-2-nitrophenol.

The diazotization method could be used to estimate either of the two aminonitrophenols separately. But the diazo dyes that were produced did not differ sufficiently to enable one amine to be detected in the presence of the other. The characteristics of the absorption curves are given in Fig. 3.

It is seen that both dyes have very nearly the same intensity of colour and the maximum absorption occurs at the same wavelength. Magne *et al.* (1932) had detected 2-amino-4-nitrophenol in the freshly voided urine of workers exposed to DNP dust. Their method was a modification of the Derrien test in which the solution was diazotized, coupled with alkaline 2-naphthol and the resultant dye extracted into ether. After addition of acetic acid and zinc ions to the ethereal solution, the 2-amino-4-nitrophenol was identified by the production of a pink colour. Under the same conditions the 4-amino-2-nitro compound produced a yellow colour. The authors quoted above used this method for the examination of the liver of a dog which had been poisoned by DNP, and in this case they concluded that 4-amino-2-nitrophenol was present.

When the Derrien test was used to examine liver homogenates, a yellow colour was produced. This was an indication of the presence of 4-amino-2-nitrophenol, but was not conclusive evidence for the absence of the isomer. Excess of 4-amino-2-nitrophenol could hide the presence of smaller amounts of 2-amino-4-nitrophenol.

A mixture of the pure compounds was added to an alumina column. On development with ammoniated ethanol, a yellow band first appeared which very soon moved well ahead of the bulk of the coloured compounds remaining at the top of the column. This yellow compound was eluted and identified as DNP. The two aminonitrophenols did not separate completely. The 4-amino isomer moved rather faster than the 2-amino compound but became diffuse. The result was a broad band which was pink at the bottom and orange at the top, the pink compound being 4-amino-2-nitrophenol. However, by paper-partition chromatography, all three compounds could be separated. The R_f of DNP was 0.60–0.65, for 2-amino-4-nitrophenol it was 0.42–0.44 and for 4-amino-2-nitrophenol it was 0.30–0.32. For the purpose of identifying and isolating the metabolic product of DNP, the two chromatographic methods were combined. An ethanolic extract of liver homogenate after incubation with DNP was added to an alumina column. A yellow band of unchanged DNP appeared and was eluted. This was followed by a pink band. Finally, after elution of the pink band, there remained at the top of the column an orange band which could only be removed after increasing the water content of the developing fluid to about 20% and it was found that this fluid did not contain a diazotizable amine.

The pink fraction obtained from the alumina column produced an orange solution after elution. This solution was evaporated to dryness in an atmosphere of nitrogen and the dry residue dissolved in 2–4 ml. of ethanol. Approximately 20 μ l. of this

solution was placed upon a paper strip and developed. After completion of the chromatogram three spots were seen: a yellow spot of the same R_f value as DNP; a brownish pink spot of R_f value of 4-amino-2-nitrophenol; and a faint orange spot at the position of 2-amino-4-nitrophenol. Subsequent analysis after cutting the spots from the paper revealed that 90% of the total amine content of the extract was 4-amino-2-nitrophenol and the orange spot accounted for the remaining 10%.

Fig. 4. Ultraviolet absorption of aminonitrophenols compared with metabolite. A, 0.0015% 4-amino-2-nitrophenol in 0.001N-HCl. B, metabolite washed from paper chromatogram with 0.001N-HCl. Estimated to contain 0.0012% of 4-amino-2-nitrophenol. C, 0.001% 2-amino-4-nitrophenol in 0.001N-HCl.

Further evidence of identity was supplied by spectrography. After preliminary separation on an alumina column and concentration into a small volume the amine fraction was placed on a paper strip and developed. The compound of the R_f value of 4-amino-2-nitrophenol was cut from the paper and dissolved in 0.001N-hydrochloric acid. Its ultraviolet absorption spectrum was observed and compared with that of 4-amino-2-nitrophenol and of 2-amino-4-nitrophenol (Fig. 4). It is seen that the absorption curve of the metabolite of DNP strongly resembles that of 4-amino-2-nitrophenol.

Thus from the evidence of chromatography and spectrography it is concluded that 4-amino-2-nitrophenol is the main reduction product of DNP in liver homogenates.

The relationship between the rate of DNP destruction and the rate of formation of amino compounds was next investigated. An experiment was made in which the amount of DNP destroyed was measured together with the amount of amine produced. The latter, calculated as 4-amino-2-nitrophenol, was determined in the trichloroacetic

acid supernatant and was thus equivalent to the total amine. It was observed that during the early stage of rapid removal of DNP there was a rapid increase in the concentration of amino compounds, but that after 3 hr. loss of amine occurred while DNP destruction continued (Fig. 5).

Earlier in this paper it has been noted that the bulk of the amino compounds were extracted by ether from acidified solutions. However, examination of the residual aqueous solution showed that some amine was still present. This compound was

Fig. 5. Comparison between DNP destroyed and amine produced by liver homogenate. Initial DNP concentration 100 µg./ml. homogenate. Dotted line is theoretical amount of 4-amino-2-nitrophenol to be expected calculated from DNP destroyed assuming 100% recovery and no destruction of amine. ○—○, DNP destroyed; ●—●, amine produced.

not extractable by ether, benzene or light petroleum, behaving in this respect as 2:4-diaminophenol. This fraction was termed the 'ether-insoluble' fraction. A similar experiment to that described above was performed with the additional elaboration of extracting the trichloroacetic acid solution with ether. The ether-soluble and ether-insoluble fractions were then analysed for amino compounds. Fig. 6 shows that as the DNP is destroyed the ether-soluble amine at first increases rapidly, but later decreases in concentration. At the same time there is a slow increase of the ether-insoluble amine the level of which is maintained after the concentration of the ether-soluble amine begins to fall. The decrease of the ether-soluble fraction commences after 3 hr. incubation. Thus it would appear that 4-amino-2-nitrophenol is itself destroyed.

The destruction of the two aminonitrophenols was next investigated. In turn, each of the above compounds was incubated with liver homogenates.

At intervals samples were removed and analysed for ether-soluble amine and ether-insoluble amine. The results of these experiments are shown in Figs. 7 and 8. Both compounds were destroyed by homogenates but at different rates. The 2-amino-4-nitrophenol is slowly destroyed with a gradual accumulation of the ether-insoluble compound. 4-Amino-2-nitrophenol is more rapidly removed. In 5 hr. very little of the ether-soluble 4-amino-2-nitrophenol is left, but there is only a slight increase of the water-soluble compound.

Fig. 6. Comparison between production of ether-soluble and ether-insoluble amine during course of DNP destruction. Initial concentration of DNP, 100 µg./ml. (Trichloroacetic acid supernatant fractionated and analysed as described in text.) Results read as 4-amino-2-nitrophenol. ○—○, ether-soluble amino compounds; ●—●, ether-insoluble amino compounds.

That the mechanism of this destruction was similar to the destruction of DNP and might possibly involve the same enzymes was demonstrated by experiment. It was found that the destruction of the amino nitro compounds could be inhibited by heat and cyanide ions but not by sodium azide, sodium malonate, *p*-chloromercuribenzoic acid or iodoacetic acid. Strict summation experiments were difficult to perform as the product of DNP reduction, 4-amino-2-nitrophenol, could not be distinguished by the method of estimation from the added amine. Moreover, the 4-amino-2-nitrophenol is itself destroyed by the liver homogenate and might also compete for the enzyme system. An indication that the presence of amino-nitrophenols lowers the rate of DNP destruction and may be competing for the enzyme system was obtained by the experiment illustrated in Fig. 9. Three quantities of homogenate were prepared. The first contained DNP, the second DNP and 2-amino-4-nitrophenol and the third DNP and 4-amino-2-nitrophenol.

Samples were taken from each homogenate at intervals of 15 min. It is seen that both compounds inhibit the destruction of DNP, but that 4-amino-2-nitrophenol has the greater effect.

Fig. 7. Rate of destruction of 4-amino-2-nitrophenol in liver homogenate. Initial amine concentration of 100 µg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 4-amino-2-nitrophenol; ●—●, ether-insoluble amine.

Fig. 8. Rate of destruction of 2-amino-4-nitrophenol in liver homogenate. Initial amine concentration of 100 µg./ml. Trichloroacetic acid supernatant fluid analysed as described in text. ○—○, 2-amino-4-nitrophenol; ●—●, ether-insoluble amine.

It has been shown that DNP is reduced *in vitro* by animal tissues to 4-amino-2-nitrophenol with only a slight amount of 2-amino-4-nitrophenol. It was, therefore, of interest to compare this result with the urine excretion products of rats poisoned by DNP. Male rats of 200 g. weight were given 20 mg. DNP/kg. body weight by subcutaneous injection.

The animals were kept in metabolism cages for the next 24 hr. and their urine collected. The urine was extracted in the same way as described for the homogenates. The dried extract was dissolved in a few drops of ethanol. Approximately 20 µl. of the extract was then developed on a paper chromatogram and compared with a known solution containing DNP and the two isomeric aminonitrophenols. Two spots were obtained from the urine extract. One had an R_F value identical with the spot due to pure DNP. The second spot was orange in colour and had the R_F value of 2-amino-4-nitrophenol. There was no sign of 4-amino-2-nitrophenol.

Fig. 9. Influence of aminonitrophenols on rate of destruction of DNP. ○—○, normal homogenate containing DNP, at initial concentration of 100 µg./ml.; ●—●, homogenate containing DNP at 100 µg./ml. + 2-amino-4-nitrophenol at 200 µg./ml.; x—x, homogenate containing DNP at 100 µg./ml. + 4-amino-2-nitrophenol at 200 µg./ml.

It has been shown that the latter compound is more rapidly destroyed *in vitro* than its isomer. It might be expected, therefore, that 2-amino-4-nitrophenol would appear as the predominant reduction product of DNP in the urine.

It has been remarked above that the 2-amino isomer was the compound found in the urine of men and dogs poisoned by DNP (Magne *et al.* 1932). It is of interest to note that the latter workers found 4-amino-2-nitrophenol in the liver of a DNP-poisoned dog.

DISCUSSION

The enzymic reduction of aromatic nitro groups is well known. Westfall (1943) and Bueding & Jolliffe (1946) have demonstrated that trinitrotoluene (TNT) is reduced by the succinic oxidase system to an amino derivative. The nitro group of chloromycetin is reduced by bacterial enzymes (Smith & Worrel, 1949) and has been related to a nitrite

reductase (Egami, Ebata & Sato, 1951). Greville & Stern (1935) showed that a bacterial succinic dehydrogenase would reduce 2:4-dinitrophenol. The present paper confirms that animal tissues can reduce one nitro group of DNP to an amino group. The process is similar to that described for TNT inasmuch that substrates of dehydrogenases, lactate and succinate, increase the rate of reduction. However, it was not possible to reduce DNP by a preparation of heart-muscle succinic oxidase. In this connexion, it may be noted that Bueding & Jolliffe did not obtain a reduction of TNT unless coenzyme I was added. Further work is necessary to see if there is any relation between the reduction of DNP with the respiratory activity of the tissue.

The next stage of the process, destruction of the amine, appears to be similar in properties to the first stage as both are inhibited by cyanide ions and not by the other enzyme inhibitors mentioned. It is possible that the reduction of the nitro group in the 4 position is followed by reduction of the 2-nitro group. Pure 2:4-diaminophenol is not extracted from water by organic solvents and would therefore be contained in what is here termed the 'ether-insoluble' fraction. Definite identification of this fraction as 2:4-diaminophenol has not been made.

It is interesting that the amino compound appearing in the urine of animals poisoned by DNP is the 2-amino-4-nitro compound. The experiments reported above show that this compound is destroyed less rapidly than its isomer. The excretion

products in the urine represent the balance of production and destruction of both aminonitrophenols.

SUMMARY

1. 2:4-Dinitrophenol is reduced by rat-liver homogenates to 4-amino-2-nitrophenol. Some 2-amino-4-nitrophenol is obtained (about 10 % of the total amine).

2. Maximum rate of reduction of 2:4-dinitrophenol is attained in phosphate buffer of pH 7 at 37° and in the presence of sodium lactate or sodium succinate.

3. The reaction is completely inhibited by 5×10^{-3} M-potassium cyanide.

4. Rat spleen, kidney, intrascapular fat, muscle and brain also destroy 2:4-dinitrophenol.

5. The reduction products were isolated by means of alumina chromatograms and paper-partition chromatography. 4-Amino-2-nitrophenol was identified by ultraviolet spectrography.

6. Liver homogenates can reduce the isomeric aminonitrophenols. The second reduction product is thought to be 2:4-diaminophenol, but has not been identified.

7. The main excretion products of 2:4-dinitrophenol in the urine of rats were unchanged 2:4-dinitrophenol and 2-amino-4-nitrophenol.

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