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THE EFFECT OF 2,4-DINITROPHENOL AND RELATED COMPOUNDS ON BILE SECRETION

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SUMMARY

1. 2,4-dinitrophenol, 2,4-dinitrophenetole, 2,4-dinitronaphthol, 4,6-dinitro-*o*-cresol, and to a lesser extent picric acid, produced an increase in bile flow and a rise in body temperature in the anaesthetized dog. The total biliary bromsulphalein (BSP) excretion in unit time was either slightly reduced, increased or remained at its pre-injection level.

2. Picramic acid, the nitrochlorophenols and 2,4-dinitrobenzaldehyde caused a moderate increase in bile flow without an effect on the temperature of the animal.

3. The three mononitrophenols, the five remaining isomeric dinitrophenols, isopicramic acid, the aminonitrophenols, phenol, 2,4-dinitroanisole, 2,4-dinitrobenzoic acid, 2,4-dinitrobenzene sulphonic acid, 2,4-dinitroresorcinol and 4-nitracatechol had little effect on bile secretion or body temperature.

4. It thus appears that, in order for a compound of this type to have a pronounced effect on bile secretion, it is necessary to have nitro groups in positions 2 and 4 of the benzene ring, and a free or potential hydroxyl group.

INTRODUCTION

It has been shown (Stone, 1959) that 2,4-dinitrophenol produces a prolonged choleresis and a marked pyrexia in the anaesthetized dog. Woo & Hong (1963) reported that injections of 2,4-dinitrophenol (2-4-DNP) resulted in a choleresis accompanied by increased excretion of bromsulphalein (BSP). This was suggested to be due to a washout of dye from the hepatic cell, a view which they stated was inconsistent with the finding that biliary BSP excretion was only slightly increased in the face of the four- to fivefold increase in bile flow. Similar results were obtained by Stone (1965) who found that the choleresis produced by 2,4-dinitrophenol (5 mg/kg) was accompanied by a marked fall in the biliary BSP concentration but that the

total BSP excreted in unit time remained either at its pre-injection level or was slightly increased. The pyrexia produced by 2,4-dinitrophenol was not the cause of the choleresis as hyperthermia produced by extracorporeal warming of experimental animals to 42° C resulted in little or no change in bile flow or bile BSP content.

The present study was undertaken in order to investigate more fully the effects of 2,4-dinitrophenol and related compounds on biliary excretion of water and bromsulphalein. A preliminary account has been presented to the Physiological Society (Brindley & Stone, 1964).

METHODS

Dogs of both sexes were fasted overnight and anaesthetized with pentobarbitone sodium; the cystic duct was ligated and the gall-bladder drained. A splenectomy was performed to ensure that the circulating blood volume remained relatively constant during the experiment. The trachea, common bile duct, ureters and thoracic duct were cannulated and bile, urine and lymph were collected serially as 10 min samples. A catheter was inserted into the left carotid artery for serial sampling of blood and the right jugular and right femoral veins were cannulated.

After a control period of 30 min, during which 10 min samples of bile, urine and lymph were collected, a priming dose (5 mg/kg) of BSP was injected into the right jugular vein and sampling continued. At the same time a continuous infusion of BSP at a constant rate was begun, the rate of infusion being such as to ensure a rising plasma concentration. Approximately 4 mg/min of BSP was infused into an 'average' sized dog (10–15 kg). Above this weight range a slightly increased dose was given.

After a period of 90 min, the appropriate phenol (2–20 mg/kg) was injected in a single dose via the right jugular vein and sampling continued for a further 2 hr. The plasma volume was determined at the end of each experiment by a dilution method using Evans Blue.

The compounds were dissolved in the minimum quantity of water and alkali and buffered with 0·1 M-phosphate, pH 7·4. 2,4-dinitroanisole and 2,4-dinitrophenetole, neither of which are soluble in this system, were dissolved in propylene glycol.

In some experiments oxygen consumption was determined using a rebreathing spirometer, blood pressure was recorded with a mercury manometer, respiration was recorded with a modified Sherrington stethograph and urine, bile and lymph flows were recorded with separate photo-transistor drop counters operating Thorp impulse counters. All these parameters were displayed on a Palmer Kymograph. These recordings were made in at least one instance of each compound tested.

BSP was determined spectrophotometrically at 580 nm in bile, urine, lymph and plasma after appropriate dilution and alkalinization. Bile samples were chromatographed on Whatman No. 1 paper, using butanol:acetic acid:water (4:1:2:v/v) as solvent in either an ascending or descending system. Bile was analysed for taurocholic acid by the procedure of O'Maille, Richards & Short (1965).

Compounds used are listed below:

2,4-dinitrophenol; 2,5-dinitrophenol, 2,6-dinitrophenol, picric acid, 2,4-dinitrophenetole; 2,4-dinitrobenzoic acid; 2,4-dinitronaphthol (BDH Ltd.). 2-nitrophenol; 3-nitrophenol; 4-nitrophenol; 2-nitro-4-aminophenol; 4-nitro-2-aminophenol; 2-chloro-4-nitrophenol; 4-chloro-2-nitrophenol; 2,4-dinitroanisole; 2,4-dinitrobenzene sulphonic acid; 2,4-dinitroresorcinol; 4,6-dinitro-*o*-cresol (Kodak Ltd.,). Picramic acid, iso-picramic acid, phenol, (Hopkin and Williams Ltd.). 2,4-dinitrobenzaldehyde; 2,4-dinitroanisole; bromsulphalein

(L. Light & Co. Ltd.). 2,3-dinitrophenol; 3,4-dinitrophenol, 3,5-dinitrophenol (synthesized in the Department). 4-nitrocatechol (synthesized in the Department of Biochemistry, University College, Cardiff).

RESULTS

The experiments were designed so that each animal should serve as its own control, and up to the time of injection of the phenol the experiments are similar, one with another. If a rising plasma concentration is to be ensured, the liver is presented with more BSP than it removes from the blood and the BSP concentration in the bile reaches a maximum for that particular dog. An equilibrium is attained, in that the amount of BSP secreted into the bile in unit time remains relatively constant. If the appropriate phenol or related compound is injected, its effects on bile flow, bile BSP concentration and bile BSP content can be noted. In those circumstances where graphic recording was employed the onset of choleresis could be timed accurately from the increased drop rate as recorded by the Thorp impulse counter. Otherwise the onset of choleresis could be observed semi-quantitatively by an increased rate of filling of the graduated tube in which bile was collected. The duration of choleresis was estimated as the interval of time from commencement of choleresis until that time when the rate of bile flow had returned to the pre-injection level.

2,4-dinitrophenol in doses of 1–5 mg/kg body wt. produced a choleresis accompanied by a decrease in BSP concentration, but the effect on total biliary BSP excretion varied; small doses (1 mg/kg) either had no effect or produced a decrease of 13 % in biliary excretion; doses of 2–3 mg/kg either had no effect or produced an increase or decrease (− 17 to 10 %) and doses of 4–5 mg/kg resulted in an increase of 8–16 % in biliary BSP excretion. The results of a typical experiment with a dose of 5 mg/kg are illustrated in Fig. 1 where it may be seen that within 10 min of giving DNP the volume of bile had increased by 200 % and that this choleresis was maintained throughout the subsequent 2 hr of the experiment. The concentration of BSP in the bile fell from approximately 8–3 mg/ml. and stayed at this low value for the remainder of the experiment. The biliary BSP content, calculated from the bile volume and bile BSP concentration, shows an interesting artifact in the 10 min sample immediately following the injection of DNP. This large increase in output is due to washout of preformed bile in the biliary tract; and this may be used to calculate the dead space of the biliary tree. The 10 min sample of bile collected immediately following injection of a choleretic has BSP present in a concentration (C mg/ml.) which may be accurately determined. This sample of bile (V ml.) is composed of dead space bile (X ml.) and freshly secreted bile (Y ml.). If it is assumed that the dead space bile has the same concentra-

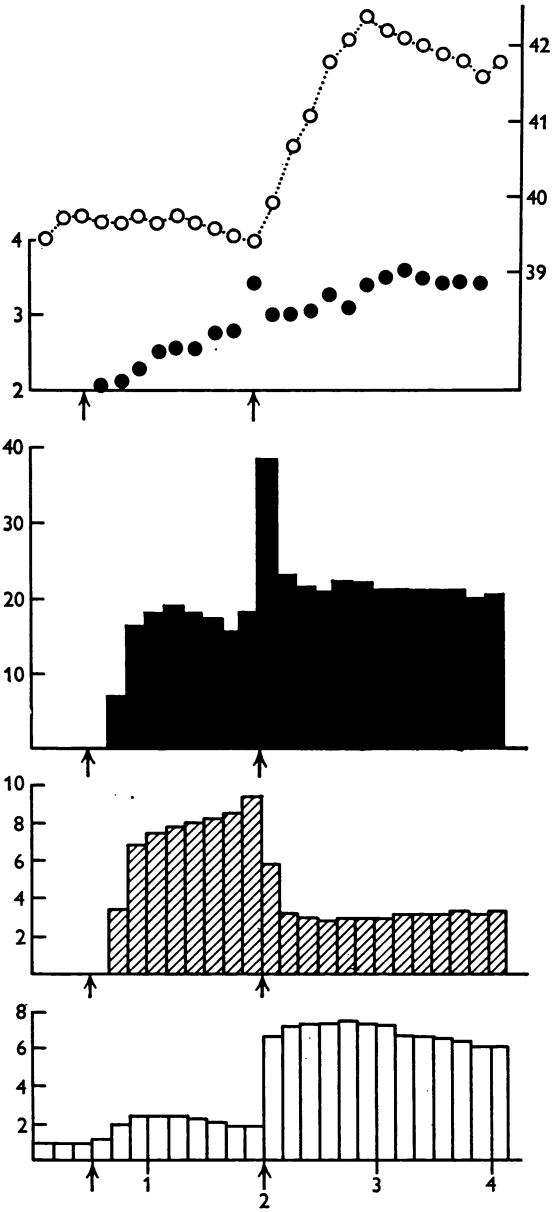

Fig. 1. Effect of 2,4-dinitrophenol on bile secretion and body temperature. Priming dose of BSP (5 mg/kg) and continuous infusion of BSP started at first arrow. 2,4-DNP (5 mg/kg) given at second arrow. Records from above downwards: Body temperature (°C), plasma BSP concentration (mg/100 ml.), bile BSP content (mg/10 min), bile BSP concentration (mg/ml.) and bile volume (ml./10 min). Abscissa time in hours.

tion as pre-injection bile (A mg/ml.) and that the freshly secreted bile has the same concentration as post-injection bile (B mg/ml.) as determined from analysis of subsequent samples, then the following relationships hold:

$$V = X + Y,$$
$$CV = AX + BY.$$

Knowing V, and determining A, B, and C the equations may be solved for X. The average value of X (biliary dead space) for twenty dogs was 1·71 ml., or expressed on a basis of volume/100 g liver weight 0·45 ± 0·04 ml. After this washout the bile content of BSP remained constant with an increase of 15 % above the control value. The body temperature of the animal rose over a period of 60 min to 42·5° C and then remained at a high value for the rest of the experiment. The results of twenty-two experiments with DNP are given in Table 1, where the essential features of the response are annotated.

TABLE 1. Effect of 2,4-dinitrophenol on bile volume, bile BSP excretion and body temperature. The values tabulated are the means and standard errors. The results for the choleresis have been calculated as the increase in bile volume expressed as a percentage of the control rate. The time of onset of the choleresis was 0–10 min after injection of the dinitrophenol. Duration of choleresis, *P*, indicates that the choleresis persisted until the experiment was terminated

Number of expts.	Dose (mg/kg)	Choleresis (%)	Duration of choleresis (min)	Change in BSP excretion (%)	Increase in body temperature (°C)
2	1	75 ± 18	*P*	−13 ± 6	1·3 ± 0·3
2	2	200 ± 30	*P*	−17 ± 8	2·4 ± 0·6
2	3	133 ± 3	*P*	+10 ± 8	2·7 ± 0·6
5	4	132 ± 7	*P*	+8 ± 6	2·9 ± 0·3
11	5	153 ± 15	*P*	+16 ± 3	3·9 ± 0·5

It may be seen that in all experiments there is a choleresis, which begins within 10 min of giving the DNP and lasts for the duration of the experiment. There appears to be no strict relationship between the magnitude of the choleresis and the dose of the compound. There was an elevation of body temperature of between 1 and 5·4° C, and this appears to be related to the dose, but there is considerable variation between individual animals. The highest temperature recorded was 44·5° C, 100 min after a dose of 5 mg/kg.

If the plasma volume is determined and the plasma BSP concentration is known, it is possible to calculate the total circulating BSP at any instant. If one adds successively the bile, urine and lymph contents, one can calculate the cumulative BSP excretion. Subtraction of the sum of plasma content and cumulative BSP excretion from the known amount of BSP infused gives the computed 'liver store' of BSP at any instant. This pro-

cedure is based on the assumption that removal of BSP by tissues other than the liver is negligible. The data of the experiment illustrated in Fig. 1 have been calculated and the results are shown in Fig. 2. It may be seen that the amount of BSP contained within the blood at any instant is but a small proportion of the BSP injected, that the cumulative biliary excretion of BSP proceeds linearly with time until the injection of DNP, and

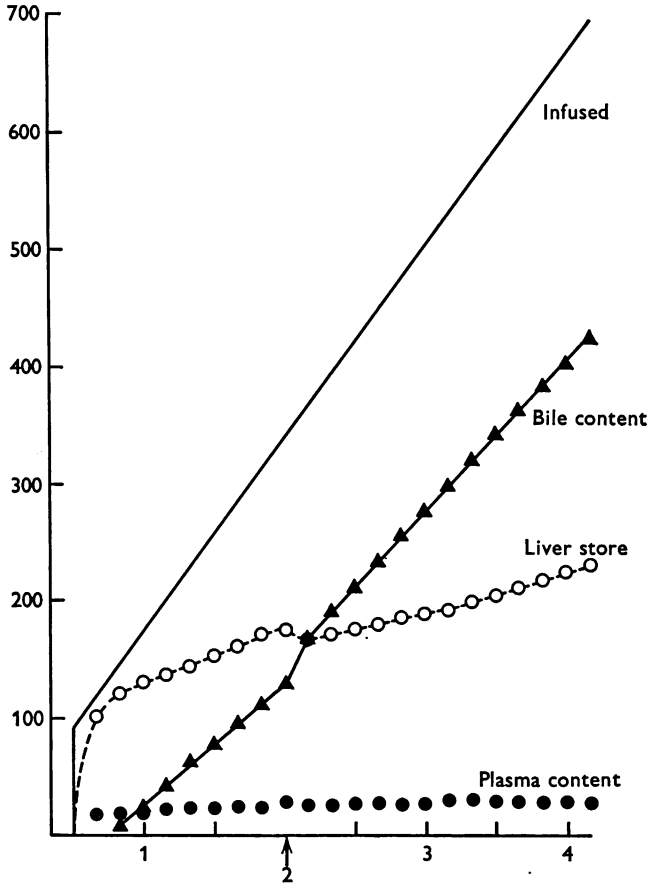

Fig. 2. Data of Fig. 1 calculated to show effect of 2,4-DNP on 'liver store' and cumulative bile content of BSP. Infused BSP (——), liver store (○ - - - ○), cumulative bile content (▲—▲) and plasma content (● ●) of BSP. Ordinate, mg. Abscissa, time in hours, 2,4-DNP injected at arrow.

that it then continues at a greater rate after the discontinuity due to washout. The 'liver store' shows a decrease after DNP, and subsequently an increase, but at a slower rate than during the control period. The liver store, as calculated, is composed of BSP within the hepatic parenchymal cell and also BSP in the biliary tree (bile canaliculi, intrahepatic bile ducts and

common bile duct). The magnitude of the fall in liver store is consistent with washout of the biliary tree and there appears to be little or no change in the BSP content of the parenchymal cell. Chromatographic analysis of the bile samples showed no significant change in distribution of conjugated and unconjugated BSP after administration of DNP.

As DNP has such a pronounced effect on bile secretion and body tempera-

TABLE 2. Effect of compounds related to 2,4-dinitrophenol on bile volume, bile BSP excretion and body temperature. The results have been calculated and expressed in the same manner as Table 1. All the compounds in this table have a free hydroxyl group and various substituents on the benzene ring

Compound	Number of expts.	Dose (mg/kg)	Choleresis (%)	Duration of choleresis (min)	Change in BSP excretion (%)	Increase in body temperature (°C)
Phenol	3	10–20	0		−7 ± 6	0
2-nitrophenol	2	5–6	10 ± 1	25 ± 5	0	0
3-nitrophenol	3	5	0		+6 ± 1	0
4-nitrophenol	3	5–6	16 ± 3	25 ± 4	0	0
2- and 4-nitrophenol	1	6	12	30	0	0
2,3-dinitrophenol	5	5–6	51 ± 13	60 ± 20	−28 ± 3	0·2 ± 0·2
2,5-dinitrophenol	4	3–5·5	50 ± 10	41 ± 26	−9 ± 9	0
2,6-dinitrophenol	3	4–5	13 ± 3	40 ± 12	−18 ± 10	0·3 ± 0·1
3,4-dinitrophenol	5	5–5·5	54 ± 14	49 ± 12	−9 ± 6	0·2 ± 0·2
3,5-dinitrophenol	4	5–6	28 ± 17	13 ± 9	−8 ± 6	0·3 ± 0·3
Picric acid	4	6–11	36 ± 14	103 ± 8	+18 ± 12	0·9 ± 0·3
Picramic acid	3	5–53	97 ± 22	*P*	−2 ± 5	0
Iso-Picramic acid.	3	5	21 ± 10	105 ± 5	−4 ± 3	0·4 ± 0·3
2-nitro-4-amino-phenol	3	5	38 ± 21	25 ± 0	−7 ± 3	0·5 ± 0·4
2-amino-4-nitro-phenol	3	5–9	35 ± 8	20 ± 5	−3 ± 6	1·0 ± 0·3
2-chloro-4-nitro-phenol	3	5–8·5	63 ± 6	90 ± 20	0	0
2-nitro-4-chloro-phenol	2	8–10	103 ± 9	*P*	+7 ± 6	0
2,4-dinitroresorcinol	1	6·7	0		0	0
4-nitrocatechol	3	5–5·5	38 ± 10	50 ± 30	−15 ± 7	0
2,4-dinitronaphthol	4	5–6	168 ± 40	*P*	−9 ± 10	1·4 ± 0·5
4,6-dinitro-*o*-cresol	4	1·5–2·5	52 ± 11	*P*	7 ± 2	0·7 ± 0·2

TABLE 3. Effect of compounds related to 2,4-dinitrophenol on bile volume, bile BSP excretion and body temperature. Data as in Fig. 1. With the exception of 2,4-dinitroanisole where the choleresis began at 33 ± 18 min the time of onset was 0–10 min. All compounds in this table have nitro groups on the benzene ring at positions 2 and 4 but the hydroxyl group has been blocked or substituted

Compound	Number of expts.	Dose (mg/kg)	Choleresis (%)	Duration of choleresis (min)	Change in BSP excretion (%)	Increase in body temperature (°C)
2,4-dinitroanisole	3	5·6–7·3	33 ± 18	43 ± 23	+10 ± 14	0·7 ± 0·3
2,4-dinitrophenetole	3	6	147 ± 18	*P*	17 ± 4	3·7 ± 0·5
2,4-dinitro benzaldehyde	3	10–12	68 ± 2	*P*	0	0
2,4-dinitrobenzene sulphonic acid	3	8–40·5	51 ± 21	32 ± 7	−5 ± 13	0·3 ± 0·3
2,4-dinitrobenzoic acid	1	10	0		0	0

ture it was of interest to determine whether these responses could be elicited by related compounds. The compounds tested are listed in Tables 2 and 3. All the compounds in Table 2, with the exception of phenol, have a free hydroxyl group, and one or more nitro groups and various substituents on the benzene ring. The compounds in Table 3 have nitro groups in positions 2 and 4 of the benzene ring, but the hydroxyl group has been either blocked by alkylation or been substituted with another group.

From Table 2 it may be seen that neither phenol, nor the mononitrophenols, nor a mixture of 2-nitro-phenol and 4-nitro-phenol has any great effect on bile volume and body temperature. Of the five isomeric dinitrophenols, none shares completely the choleretic as well as the pyrogenic action of 2,4-dinitrophenol. 2,4-DNP is metabolized in the body to 2-amino-4-nitro-phenol and 2-nitro-4-amino-phenol and neither of these compounds has a large effect on bile volume and body temperature.

Picric acid and one of its break-down products, picramic acid, produce a long-lasting choleresis, with or without a pyrexia. Replacement of a nitro group in positions 2 or 4 by a chlorine or hydroxyl group also results in a diminution of activity. 2,4-dinitronaphthol and 4,6-dinitro-*o*-cresol retain the activity exhibited by 2,4-DNP.

The only compound in Table 3 to behave similarly to 2,4-DNP was 2,4-dinitrophenetole. Although the hydroxyl group has been ethylated, this chemical produces the long lasting choleresis and pyrexia usually associated with 2,4-DNP. On the other hand 2,4-dinitrobenzaldehyde produces the choleresis without the elevation in body temperature.

DISCUSSION

The effects of 2,4-dinitrophenol on biliary secretion were similar to those reported by Woo & Hong (1963) and by Stone (1965), viz. choleresis accompanied by a fall in BSP concentration, but a rise in the amount of BSP excreted in unit time.

The three mononitrophenols and the five isomeric dinitrophenols did not show the pronounced effect on bile secretion associated with 2,4-dinitrophenol. From this, it is concluded that it is necessary to have two nitro groups on the same molecule, and they have to be in positions 2 and 4 on the benzene ring. Picric acid retained some of the properties of 2,4-dinitrophenol in stimulating metabolism, but increasing the number of nitro groups did not increase the choleresis nor potentiate the effect on body temperature. In fact, any modification of the 2,4-DNP molecule tended to decrease its metabolic effects.

2,4-DNP is metabolized in the body to 2-amino-4-nitro-phenol and 2-nitro-4-amino-phenol (Guerbet & Mayer, 1932) and both these com-

pounds had but a slight effect on bile volume and body temperature. This seems to indicate that it is 2,4-DNP, and not its metabolic products, which stimulates metabolism. The lack of, or reduced, response observed with chloronitrophenols, and 4-nitrocatechol, and the retention of the complete response with 2,4-dinitronaphthol and 4,6-dinitro-*o*-cresol support the conclusion that it is necessary to have two nitro groups on the same benzene ring.

It is apparent from Table 3 that replacement of the hydroxyl group by carbonyl, carboxyl or sulphonic acid groups produced a reduction in biological activity. Blockade of the hydroxyl group by methylation to yield 2,4-dinitroanisole also resulted in reduction in activity. However, the ethylated phenol, 2,4-dinitrophenetole retained the activity of the parent compound. The dinitrophenetole did not contain any free phenol, as judged by solubility and ferric chloride tests, and it is suggested that the compound is rapidly de-ethylated *in vivo* to form active 2,4-dinitrophenol. In contrast, it is assumed that demethylation of 2,4-dinitroanisole does not occur.

The result with 2,4-dinitroresorcinol cannot be explained. It has a free hydroxyl group on position 1, nitro groups on positions 2 and 4, and yet lacks activity. It was not that this particular animal was refractory because an injection of 2,4-DNP given at 240 min produced the usual choleresis and elevation in body temperature.

It is not possible from the present experiments to indicate the mechanism by which 2,4-DNP produces a choleresis in the dog. It has been assumed that the effect was due to the 'uncoupling' of oxidative phosphorylation, a known property of 2,4-DNP. In this situation oxygen consumption and substrate utilization are increased but the generation of high energy phosphate bonds is depressed. It was considered that ATP would be used as an intermediate in the transfer of energy from substrate to the physico-chemical processes involved in bile formation. The absence or reduction in quantity of this intermediate should lead to a cessation or modification of the rate of bile secretion. It is difficult to understand why, in the dog, the response should be an increase in volume.

In the anaesthetized rat it was shown (Schenker & Combes, 1967) that depletion of hepatic ATP to 25% of the normal concentration by administration of ethionine and 2,4-DNP did not result in a significant change in bile volume nor a significant alteration in BSP secretion into bile. Somewhat similar results have been reported by Bizard (1965) and Bizard, Vanlerenberghe, Robelet, Milbled & Guerrin (1960) using the isolated perfused rat liver except that they observed a fall in the rate of bile flow after 2,4-DNP.

If the choleretic effect of 2,4-DNP is due to its uncoupling action, then

there should exist a direct relationship between the activity of the dinitrophenols *in vivo* and their known potencies as uncoupling agents. Burke & Whitehouse (1967) have determined the relative potencies of the six isomeric dinitrophenols in uncoupling phosphorylation in rat liver mitochondria. The potencies, in declining order, are 3·5 > 2·4 > 2·6 ≏ 3·4 > 2·3 ≏ 2·5. This order does not agree with the results found in the present study where 3,5-dinitrophenol was one of the least effective compounds to affect bile volume and BSP excretion. The results presented here are however consistent with the findings of Cameron (1958) on the stimulation of metabolic rate in rats.

It has been postulated by Sperber (1959) that the primary event in the formation of bile is an active transport of bile salt from the parenchymal cell into the bile canaliculus. This would lead to the establishment of a chemical gradient, and the osmotic effect of this gradient would result in a flow of water and dissolved molecules into the bile canaliculi. The water and dissolved solute could pass through pores in the canalicular membrane which are too small functionally to permit back diffusion of bile salt. In support of this hypothesis, it has been shown in the fasting dog during cholinergic blockade that the rate of bile flow is directly proportional to the rate of taurocholate secretion (Preisig, Cooper & Wheeler, 1962). Additional evidence comes from the observation of Sperber (1965) that there is a correlation between osmotic activity and choleretic potency of several secreted compounds including bile salts.

It is unlikely that 2,4-DNP produces the increase in bile flow by this mechanism as determination of taurocholic acid in the bile shows a decrease in concentration and content during the choleresis compared with bile formed during the control period. 2,4-DNP itself cannot cause an osmotic choleresis as the largest dose given to any animal was 0·6 mM.

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