THE MECHANISM OF ACTION OF ORGANIC NITRATES

By

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FOREWORD

Hypertensive disease and its sequellae are among the chief causes of death in the United States. They are said to cause the highest disability rate of all illnesses in the age period of 25 to 64 years (67). The ultimate cause of this condition in the majority of cases has not been found. Many aspects of the phenomena associated with the disease have been studied. Much is now known concerning the physiological and pathological changes which are produced (33). However, our knowledge is still far from complete, and the treatment which has been employed has usually been unsatisfactory.

One of the most potent groups of vasodilator drugs is that of the nitrites and the organic nitrates. After nearly a century of use, disadvantages attending their use in chronic hypertension are well known.

The purposes of the present study are to explore the realm of water soluble organic nitrates, the effects of varying oil and water solubility ratios in a series of homologous nitrates, and some possible mechanisms by which the group acts. These aims are more adequately explained in Chapter III. Some aspects of the problems of hypertension are reviewed briefly. Historical developments in the field of nitrite and nitrate therapy are presented, and some current problems discussed.

CHAPTER I

PROBLEMS AND CLINICAL ASPECTS OF HYPERTENSION

Normal systemic arterial blood pressure in the human being is given values which differ among various authors. If the figures are pooled, normal systolic pressure has a range between 90 and 140 mm. mercury, and normal diastolic pressure one between 60 and 90 mm. (58). The average figures for large groups increase with the age of the individuals. Many clinicians agree that hypertension exists if diastolic blood pressure remains above 90 mm. mercury. Likewise, if systolic blood pressure remains above 140 mm., the condition exists. The diastolic level is watched more closely by internists, who often speak of "diastolic hypertension".

PHENOMENA RELATING TO HYPERTENSION AND HYPERTENSIVE DISEASE

It should be pointed out that it is the peripheral resistance which mainly determines the diastolic level, and that the aggregate cross-sectional area of the arterioles is the chief factor in the maintenance of that resistance. On the other hand, the systolic level is largely a function of the volume of blood ejected by the left ventricle at each contraction. Systolic pressure may be raised considerably without a rise in diastolic pressure in certain conditions.

In these cases, there is no increase in peripheral resistance, and no diastolic hypertension. The overwhelming majority of cases of hypertensive patients have increased arteriolar tone as the direct cause of their elevated pressure, and in them, both diastolic and systolic pressures are elevated. Yet cardiac output, rate of blood flow, and capillary and venous pressures are usually normal until complicating sequellae occur.

Neurogenic Etiology of Hypertension

The causes of the hypertonicity of the arterioles have been sought for many decades. Some of these have been found in experimental animals, and some in man. The vasoconstrictor fibers of the sympathetic nervous system normally control the state of contraction of the arterioles. As an increased outflow of the nerve impulses over those fibres normally contributes to elevation of blood pressure in times of stress, it is not surprising that this division of the autonomic system has been suspected as an etiological factor in hypertensive disease.

Resection of varying amounts of the thoracolumbar sympathetic outflow has resulted in the reduction of blood pressure to normal or near normal values in some cases of the disease. Likewise, great decrease in pressure has been obtained when the autonomic ganglia are blocked by tetraethyl ammonium chloride, (13) and when the nerve impulses are blocked more peripherally by the use of Dibenamine

(N,N-dibenzyl -β- chloroethylamine hydrochloride) (36). These procedures point to the dominating role of the sympathetic vasoconstrictor system in the maintenance of the hypertensive state in some individuals. The selection of cases for surgical treatment is based on evidence of increased neurogenic tone of the arterioles and of the absence of the fixed narrowing which may occur in arteriosclerosis and in long standing hypertensive disease. This evidence is obtained by use of drugs, such as those mentioned above, or a barbiturate in hypnotic doses. If the blood pressure cannot be lowered sufficiently by these means, surgery is not usually indicated, since experience has proved sympathectomy to be ineffective in such cases.

The role of the emotions in the production of hypertension has been partially recognized by physician and laymen for many decades. It has been extensively studied and described by physicians, psychologists, psychiatrists, and especially by psychoanalysts. The latter claim to have found a more or less specific type of subconscious emotional content in the personality of many patients with primary hypertension. This pattern is probably developed early in life in response to prolonged harshness in interpersonal relationships. The repressed feelings of resentment, fear, anger, perhaps reaching near-panic states, repeated often enough, are thought to be "stored" in the unconscious mind as a force, a potential energy, which later flows out through

the autonomic nervous system to produce increased vascular tone. The individual may be unaware of such misguided emotions in his background, and believe that he is well adjusted as an adult. Deep psychoanalysis may be the only way to determining the content of the unconscious mind, from which flow forces which affect human behavior and physiology, and which may produce disease. This is an extremely abbreviated summary of some of the concepts that have grown out of a branch of medicine which is being further explored and more widely recognized. The relationship between an individual's experiences and the production of hypertensive disease will not be proved as easily as the etiology of an infectious disease, but the psychosomatic theory seems logical to those who have grasped its dynamic role in mankind's ills.

Humoral Theory of Hypertension

Another direction which the search for the causes of arteriolar spasm has taken is the humoral one. Excess circulating epinephrine has not been found in primary hypertension, but has been found in cases of pheochromocytomata, which are epinephrine producing tumors of chromaffin tissue, embryologically related to the adrenal medulla. They are relatively rare, and excision of all abnormal tissue usually cures the disease. Other pressor amines have been searched for, particularly since the role of the ischemic kidney in hypertension was shown (32). They have not been demonstrated

to have an etiologic role in human hypertensive disease, although some experiments in animals indicate that they are involved in the hypertension produced by renal ischemia.

The outstanding humoral mechanism in renal hypertension is the renin-hypertensin (renin-angiotonin) phenomenon. Hypertensin (angiotonin) is a powerful vasoconstrictor, acting directly on the smooth musculature of the arterioles (8, 59). It has been found in very few cases of human primary hypertension, and then only in the early stage when the arterial pressure is rising. In some cases of shock and of toxemia in pregnancy (17), the renin mechanism has been demonstrated. In many cases of renal disease of various types, hypertension is produced, and, if the renal involvment is unilateral, removal of that organ is commonly followed by a reduction of blood pressure, even to normal values. Thus tumors in or near the kidney, cysts, bacterial infections, calculi, and other operable pathology may cause the renin-angiotonin sequence resulting in hypertension. Glomerulonephritis and other diffuse diseases of the kidneys may cause hypertension by the same mechanism, initially (17). Experimentally and in man, hypertension, although initially produced by the renin mechanism, may become independent of that humoral device, and become permanent even if the ischemic kidney is removed and the fellow organ is normal. The chronic spasm of the arterioles leads to irreversible fixation in the narrowed state, just as it

occurs in late primary hypertension. In such cases, reduction of blood pressure by means of tetraethyl ammonium salts, Dibenamine, barbiturate hypnosis, removal of a diseased kidney (unilateral pathology), or sympathectomy, is impossible or unsatisfactory.

Other methods of producing hypertension have been employed, such as resection of the carotid sinus and aortic depressor nerves, compression of carotid arteries with resultant cerebral ischemia, and Kaolin injections into the ventricles of the brain. There is no evidence that any of the phenomena underlying hypertension produced by the foregoing manipulations are involved in the establishment of human hypertension.

CLINICAL SYNDROMES

The percentage of cases of human hypertension for which definite cause can be found, has been estimated as low as 5 per cent (59). Thus 90 or 95 per cent can be termed "primary hypertension", or, less appropriately, "essential hypertension". The remainder is known as "secondary hypertension", and is known to be produced by a large number of conditions.

Thus most of the hypertensive patients have no proved cause for their disease, and it is likely that the causes are many and varied among such persons. Many of them suffer increasing damage to several areas of their bodies during

the years of hyperpiesis. Some have mildly elevated pressure until death results from arteriosclerosis or other cause, with little or no other complications. Some have sufficiently elevated tension as to burden the heart to the extent of producing cardiac failure. Forty four to sixty four per cent terminate in this way according to various clinician authors. Cerebral vascular accidents claim the next largest group, estimated to comprise between six and twenty per cent. In about eight per cent, renal function becomes so poor as to cause death in uremia. All of the foregoing complications can occur in the group called primary hypertension which is classified as "benign" in contradistinction to a small class called "malignant hypertension". These two categories were advocated in 1914, and are still justified. An estimated 10 per cent of hypertensive patients will eventually develop the malignant phase of the disease. This is a rapidly progressive, fulminating accentuation of the hypertensive syndrome characterized by necrosis of arterioles in various areas, with hemorrhage, thrombosis, encephalopathy, uremia, and markedly elevated arterial tension. The diastolic may exceed 140 mm. Hg in such cases, and the systolic, 300 mm. This malignant phase accounts for nearly all deaths from renal damage in the course of hypertensive disease.

INADEQUACY OF KNOWN METHODS OF TREATMENT

Surgical measures

Results of extensive bilateral extirpation of the thoracicolumbar sympathetic nerves shows that satisfactory reduction of diastolic pressure is seen in a varying percentage of cases, according to reports from various clinics. Fishberg, in 1948 (27), reported a well studied series of 119 hypertensive patients selected for sympathectomy with a follow-up period averaging 32 months. A 25 per cent or greater reduction of diastolic pressure was still present in only 3o of the cases, although "worthwhile symptomatic improvement" occurred in 59 per cent. Headache was often relieved. Retinopathy, present in 17 cases before operation, was greatly improved in 12. Fishberg believes that improvement in these cephalic manifestations may result from the redistribution of arterial blood, a slightly smaller amount going to the head than before operation. Symptomatic improvement may occur without a fall in blood pressure after sympathectomy. Several clinicians now consider this surgery a palliative and not a curative procedure, and Fishberg concludes that it is indicated in less than 4 per cent of patients with "essential" hypertension.

Medical management

The medical treatment of hypertensive disease has been even less effective. Sedatives have but limited value.

Vasodilators have been used for three quarters of a century, and are still widely employed. However, in most clinical reports, they receive little or no praise. Despite timely reports to the contrary, potassium thiocyanate has fewer and fewer advocates among internists. Most of the careful evaluations show it to be ineffective. The nitrates, although some are potent depressor agents, are apparently not popular with leading clinicians. Yet many physicians prescribe nitrates as well as thiocyanate. Potassium iodide and other agents are also used in hypertension, but it must be concluded that the profession recognizes no drug of real value for the continued reduction of arterial tension.

Sedative doses of a barbiturate, xanthine derivatives, attempts at personal readjustments of home and work factors, and varying amounts of psychotherapy, probably constitute the main regimen of rational treatment. Properly carried out, these measures provide some degree of success in most individuals, at least intermittently. There is still need of a more reliable vasodilator when the foregoing therapeutic aids do not suffice.

Tetraethyl ammonium salts are not indicated for continued treatment, nor is "Dibenamine". The homeostatic function subserved by the sympathetic system is of such importance, even in the hypertensive patient, in whom that system may be overactive, that paralysis of it must not be

recklessly accomplished. The organism is at a distinct disadvantage if it cannot quickly adjust itself to the sudden changes caused by postural, post-prandial, emotional, exertional, and other physiological factors. The sympathectomized patient frequently has distressing postural hypotension requiring an abdominal binder to resist the graviation of blood into the splanchnic area and legs in the upright position.

Thus for physiologic reasons, the control of arteriolar tone by means of nerve blocking drugs or procedures is a complicated problem. Yet this avenue of approach is aimed at one of the direct causes of the vascular hypertonicity, and may eventually offer a satisfactory therapeutic application. We must learn to reduce the excessive and usually futile outflow of nerve impulses to the vessels, yet to allow the physiologic stream to flow and fluctuate normally. That would appear to be a difficult feat to expect from a pharmacologic agent. While its accomplishment is being awaited, a directly acting vasodilator drug might offer some advantages. There also remains the possibility that humoral substances are involved in the maintenance of hypertension.

Drugs which act upon the smooth muscle cells of arterioles, causing relaxation without paralysis, may yet constitute the most satisfactory adjunct to the treatment of hypertension. Physiologic vasoconstriction is possible

during the residence of the nitrates in the body in most cases, hence function is not too greatly handicapped. In order to be useful, however, such drug must be able to relax the muscularis most of the time, and with a sufficient degree of action as materially to lower the diastolic pressure. Furthermore, the toxicity must be low enough to allow continued use. Tolerance must not be too rapidly developed, although slow development would not prohibit the use of the agent in intermittent periods alternating with a few weeks in which sensitivity might be restored. Even intermittent therapy might be better than none, and some patients might be slow to develop tolerance. Low toxicity would allow gradual increase in dosage as tolerance develops.

It was with these physiologic and pharmacologic principles and aims in mind that the present research was executed.

CHAPTER II

HISTORY AND PHARMACOLOGIC ACTION OF NITRITES AND OF ORGANIC NITRATES

GENERAL SURVEY

The first nitrite mentioned in the literature, according to Atkinson (2), is that of ethyl, the discovery of which is accredited to Raymond Lully (1235 to 1315). It was the only nitrite used in medicine until the middle of the nineteenth century. Richardson reported on its toxic manifestations in 1867 (66). Mention has been made, however, of the incorporation of potassium nitrate in "niter papers" for the treatment of asthma in 1842. The powder in these packages was burned and the smoke inhaled. Any nitrite formed and absorbed by the lungs might cause relaxation of the smooth muscle fibres of the bronchioles. Such remedies survive even today, but are not officially recognized.

Modern therapy with this class of substances began with the pharmacological investigation of glyceryl trinitrate by Pelikan (6), and by Field (24), in 1858. This compound was first prepared by Sobrero in 1846. In 1859 Guthrie (34) demonstrated some of the actions of amyl nitrite[1] upon

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[1]Actually an impure mixture of isoamyl, 2-methyl butyl, and other nitrites.

the inhalation of its vapors. He reported observing an acceleration of the pulse, throbbing of the arteries in the neck, and flushing of the skin of the face and neck in a group of students. These are symptoms noticed by most patients if enough of the vapor is inhaled. Amyl nitrite was prepared by Balard in 1844 (4).

Brunton studied the pharmacology of the compound in 1870 (9) and used it in a case of angina pectoris with gratifying results (10). Richardson began using the substance in the same condition, and soon it became a widely known remedy. Glyceryl trinitrate had been used in medicine by Herring (4), a homeopathic physician, even earlier, but not apparently for angina pectoris. Its use in this condition soon followed the introduction of amyl nitrite. Both of these substances are used extensively today in the treatment of angina with very good results. Their mode of action in relieving the pain of cardiac ischemia was not understood when their use was inaugurated. Brunton knew that the systemic arterial blood pressure was reduced after inhalation of amyl nitrite, and since his patients in attacks of angina had hypertension which was lowered by the treatment, he assumed the relief of pain was produced by the decrease in the resistance against which the heart was working.

Although the exact mechanism of action in this condition was not recognized, the drugs were introduced into therapeutics because they had already been known to have a

definite physiological action, namely that of lowering the blood pressure. Dunstan (22) in 1888 noted that amyl nitrite was one of the few drugs ever to have been thus introduced. The empiricism, which has characterized the early use of the majority of medicinal agents, is absent from the history of these indispensable drugs.

The elucidation of the modus operandi of these two compounds began shortly after their usefulness was demonstrated, and continues today with many questions unanswered. The dramatic actions of the drugs early engaged the interest of leading physicians and physiologists, mainly in England and Germany. The more important pieces of work in the development of the pharmacology of this class of compounds will be briefly reviewed. A more extensive coverage of this subject has been recently published by Von Oettingen (72) in a monograph with a large bibliography.

In order to determine the site of action of amyl nitrite in lowering the blood pressure, Brunton (9) administered the vapors to animals after section of the cervical spinal cord. Definite depressor responses ensued. He concluded that the drug acts on the muscular walls, either directly on the muscle fibres or through the nerve endings. This explanation was opposed by Bernheim (6) who gave enough of the nitrite to produce convulsions with the hypotension, and postulated the theory that the drug reduces blood pressure by action on the vasomotor center. He believed that

Brunton had failed to cut all spinal tracts, and thus obtained the depressor response by central action.

Another theory of action was brought forward by Wood (75), who explained that the venous blood was caused to "stay dark" by nitrite. The formation of an altered form of hemoglobin by nitrite was demonstrated earlier by Gamgee (31). Wood believed that the prevention of oxidation processes through that mechanism would certainly affect all tissues, and that lowering of blood pressure was an example of a general functional disturbance. He observed that phosphorus glowing at 112°C. would suffer extinction of the oxidation by vapors of amyl nitrite. He considered this effect as a clue to the physiological action.

Brunton's first conclusion, that the muscle fibres themselves are the site of action of nitrites has been amply confirmed by subsequent work. The amyl nitrite and the trinitrate of glycerol were so useful that their pharmacology was studied extensively.

The similarities in the actions of amyl nitrite and potassium nitrite were emphasized by Reichert and Weir-Mitchell in 1880 (65). They wanted to demonstrate that it is the nitrite group that is primarily if not solely responsible for the characteristic actions of the former drug, and not the amyl portion of the molecule. It was observed that amyl alcohol caused moderate depression of blood pressure.

Sodium nitrite was used in the treatment of angina pectoris by Hay in 1883 (37) with some degree of success. It is not as rapid in relieving the pain, however, and its use in that condition did not survive. Hay studied other types of nitrites and nitro compounds (38), finding no other promising ones.

In 1888, after nearly thirty years of investigation and clinical use, amyl nitrite was exposed by Dunstan (op. cit.) as being a heterogeneous mixture. It was prepared from impure "amylic alcohol" obtained from fusel oil by fractional distillation. The description and manner of preparing and of analyzing amyl nitrite set forth in the British Pharmacopoeia had allowed such heterogeneity of composition. The drug contained two isomeric amyl nitrites, each a branched chain, and very difficult to separate, and probably small amounts of isobutyl, propyl, and ethyl nitrites, depending upon the number of refractionations. Dunstan prepared the α-isoamyl and β-isoamyl nitrites separately and found them to be of nearly equal potency. Isobutyl nitrite was more potent than either.

Cash and Dunstan continued the comparison of members of the aliphatic nitrites and published in 1894 a lengthy report (12) on some of the actions of the nitrous acid esters of all possible saturated aliphatic alcohols of one to four carbon atoms, and of three branched chain amyl alcohols. These nitrites were compared on the basis of

the inhalation of equal volumes. The variation of the molecular weights was not considered properly. For this and other reasons no complete and accurate picture of the effect on physiological activity of lengthening and branching the carbon chain can be drawn. Five of the members of the series gave deeper falls in blood pressure, and six gave more prolonged falls than did the isoamyl nitrites. Yet the latter continued to be the only volatile nitrite to be used by inhalation. Its precedence and the availability of the amyl alcohols probably account for its supremacy even today. Cash and Dunstan did not use methods which would clearly establish relative potency. Bioassay of such potent drugs is a difficult problem, and it seems doubtful that these authors arranged them in the correct order of activity. An attempt in the present work has been made to compare relative potency in a homologous series of nitric esters. The difficulties involved in such assay procedures will be discussed in connection with that work.

A number of new aliphatic nitrites was studied by Krantz, Carr, and Forman (44, 45, 48), the synthesis of some of which was reported by Forman, Carr, and Krantz (29). They explored the action of saturated members having up to and including 18 carbon atoms, including branched chains, when inhaled by anesthetized dogs. Potency as depressors diminished as the molecular weight increased, esters having more than ten carbon atoms being almost ineffectual. The

vapor pressure of these nitrites was related to their potency, and was shown to diminish rapidly as the chains were lengthened from 5 to 8 carbon atoms, and to be quite low in all higher members. Increasing the molecular weights of shorter chain nitrites by introducing bromine atoms (44) provided longer action, but the nasal mucosa was too greatly irritated by the vapors of such brominated compounds to warrant their clinical use.

One of the branched chain compounds studied by these authors, 2-ethyl-n-hexyl nitrite (45), was found to produce depression of the dog's systemic blood pressure for much longer periods than amyl nitrite. In normal persons the effect was over by the sixth minute. The authors found the production of methemoglobin to be less with this compound than with amyl nitrite. It was found by Freedberg, Spiegel, and Riseman (30) to be effective in the treatment of angina pectoris, and by Field (25) to be superior to amyl nitrite and to glyceryl trinitrate in the treatment of spasm of the cardiac sphincter of the stomach.

The pharmacology of the inorganic nitrites was studied further by Atkinson (op. cit.) and Leech (50, 51). They showed conclusively that the effect of the -O-N=O radical is the relaxation of smooth muscle by direct action on the fibres. All smooth muscle is so affected. With regard to skeletal muscle, Leech showed that sodium nitrite progressively weakens the contractions of the frogs gastrocnemius

in response to electrical stimulation. The rapidity of the paralysis is apparently a function of the concentration of the drug. A solution containing 0.75 per cent $NaNO_2$ kills a muscle in 15 minutes; one containing 0.1 per cent kills in 30 to 40 minutes; and one of 0.005 per cent in 24 hours. Leech states that moderate nitrite effects are reversible.

Leech demonstrated the accelerating and weakening effects of sodium and amyl nitrites on the isolated frogs heart. These may also be reversed by washing the drugs out with nitrite-free perfusion fluid (saline or blood). Acceleration of the mammalian heart is an outstanding feature of nitrite action. It has been shown to be largely reflex in origin, resulting from decreased blood pressure.

Nitric acid esters of monovalent alcohols have been found to be unsuitable in therapeutics because of their weakness as vasodilators and becuse of prominent toxic reactions. Nitrates of polyvalent alcohols are much more potent, and several of them have found extensive use as depressor agents. In 1895, Bradbury (7) compared the efficacy of glycol dinitrate, glyceryl trinitrate, erythrityl tetranitrate and mannitol hexanitrate. He injected them into the stomach of animals, rabbits usually, and took tracings of the blood pressure. The extreme degree of insolubility of the latter two compounds let him to use larger doses which were incompletely absorbed. These two gave depression of blood pressure of more prolonged

duration, although of somewhat lesser degree.

Matthew (55) studied the depressor action of the various nitrites and nitrates in hypertensive patients and found their depth and duration of effects to vary widely. The longest acting drug was again found to be mannitol hexanitrate. It is the most insoluble member of the group of polynitrates. It is being used extensively today in attempting to lower high blood pressure, but not by all physicians. It is given orally in doses of 16 to 32 mg. and repeated at four to six hour intervals. Glyceryl trinitrate is active in an oral dose of 0.6 mg. and is only soluble to the extent of 0.18 per cent in water. This dose may also be given hypodermically, or may be absorbed from the floor of the mouth. These avenues are usually used in the anginal attack.

THE PROBLEM OF THE REDUCTION OF ORGANIC NITRATES TO THE NITRITE ION AS THEIR MODE OF ACTION

After the similarities in the actions of amyl and inorganic nitrite were described by Reichert and Weir-Mitchell (op. cit.), many authors held the view that both substances acted through the medium of nitrous acid derived from their hydrolysis. Aqueous solutions of amyl nitrite were always found to be acid in reaction by Leech, suggesting hydrolysis. Nitrous acid has never been isolated because of rapid spontaneous decomposition. Its presence in higher concentration

is indicated by a blue color in the solution, and by the liberation of brown fumes of the higher oxides of nitrogen. These characteristics are not detectable in the small amount of the acid that results from hydrolysis of a solution of amyl nitrite. A saturated solution of the latter contains a very small quantity of the ester.

The pharmacologic action of glyceryl trinitrate was early suspected as being produced by its decomposition products. This was suggested by Onson in 1865. Hay (39), in 1883, reported the appearance of a trace of nitrite after incubation of glyceryl trinitrate with blood. He also demonstrated the conversion of approximately two thirds of the nitrogen of that compound to inorganic nitrite by alkali. He was impressed by the similarities in the actions of this nitrate and sodium nitrite. Cagnoli (11) and Atkinson (op. cit.) confirmed the liberation of nitrite from glyceryl trinitrate in vivo. Many modern text books give the explanation that the nitrates act because of the liberation of the nitrite ion. It is usually assumed that this liberation occurs in the blood or liver, and that circulating inorganic nitrite is then responsible for the pharmacologic actions.

In 1899, Marshall, in his doctorate thesis, discussed recently by him (54), presented evidence that suggested that glyceryl trinitrate acts as such and not through liberation of nitrite ion. He washed the vascular system of a

terrapin with saline acidified with acetic acid (1 in 60,000) in order to remove any possible alkaline effect on hydrolysis of the nitrate. Vasodilation was obtained when glyceryl trinitrate was added to the perfusion fluid. He found no nitrite in the perfusate, using a test sensitive to one part in two million. Other nitric esters gave the same negative results.

Crandall, Leake, Loevenhart, and Muehlberger (16) observed in 1929 that glyceryl trinitrate disappears very rapidly from the blood, only about 14 per cent remaining in 1 minute. Nitrite was detected qualitatively. Crandall (15) later reported that various tissues destroy glyceryl trinitrate in perfusion fluid, with the appearance in the perfusate of varying amounts of nitrite. Blood was found to affect the nitrate similarly, the action being due entirely to the erythrocytes. Marshall states that "saponification" of glyceryl trinitrate proceeds very slowly at the pH of blood, whereas immediate vasodilatation results when the drug is injected intravenously. He further argues that methyl nitrate, which is not hydrolyzed to nitrite by alkali (54), causes an immediate fall in blood pressure on intravenous injection.

Marshall was one of the first to point out the difference in the quantitative responses of glyceryl trinitrate and sodium nitrite in perfusion experiments in warm blooded animals. The nitrite was very much weaker in eliciting

vasodilatation in sheep kidneys than the organic nitrate. It is thereby suggested that it is not circulating blood nitrite which may be liberated by organic nitrates that accounts for the pharmacologic response. In an earlier paper (52), 1897, Marshall wrote:

> ..... and from my own observations I am inclined to think that nitroglycerine acts as such. At least any transformation that occurs is brought about in the tissue cells themselves. Here an initial reduction to sodium nitrite probably does occur; in any case the decomposition changes proceed along the same lines as with the nitrite, and as a result a similar effect is produced.

Herrman, Leake, Loevenhart, and Muehlberger (41), in 1926, showed that with methyl nitrate, ethylene glycol dinitrate, glyceryl trinitrate, and mannitol hexanitrate, there is a parallel relationship between depressor potency and rate of alkaline hydrolysis. This suggests that organic nitrates may act by liberation of inorganic nitrite. It should be mentioned that the very stable inorganic nitrate does not lower blood pressure.

An organic nitrate quite resistant to alkali hydrolysis, producing a negligible amount of inorganic nitrite in 0.1 N NaOH at 37° in 1 hour was prepared by Forman, Carr, and Krantz, (op. cit.) and studied by Krantz, Carr, Forman and Ellis (48, 49). This compound, isomannide dinitrate, is a potent vasodilator, yet when injected intravenously did not produce detectable nitrite in the blood. Rath and Krantz (54) followed the blood nitrite level after injection of

sodium nitrite, and found values immediately following the administration which were much lower than anticipated. The blood pressure was immediately lowered by the injections. It remained low for approximately one hour, although the nitrite content of the blood fell rapidly. These investigators found the normal nitrite content of human blood to be 9.45 gamma per 100 cc, with a standard error of 0.47.

They were able to increase the level to 90 gamma per 100 cc blood by a proper dose of sodium nitrite with no fall in blood pressure. When a 5 per cent fall was obtained by larger amounts of the drug, the blood concentration was 1,000 gamma per cent in one dog, and 330 gamma per cent in another. The traces of nitrite ion in blood found by some workers after injecting organic nitrates such as glyceryl trinitrate would appear not to be responsible for the vasodilator action, in light of the above findings. Such small quantities are incapable of producing the effect.

After administration of depressor doses of nitric esters, Krantz, Carr, Forman, and Ellis (49) and Rath and Krantz (64) failed to find any nitrite in the blood. The conclusion that depressor activity of nitrates is not caused by nitrite ions <u>in the blood</u> seems therefore valid. If all the nitrogen in an effective intravenous dose of glyceryl trinitrate could be immediately changed to nitrite ion, it would be far below the minimal effective dose of nitrite required to produce a fall in blood pressure. The same is

true for oral therapeutic doses of mannitol hexanitrate in man (32mg.).

A matter which has been studied recently is the action of intracellular systems upon organic nitrates.

It has long been known that bacteria are able to reduce nitrates to nitrites, as this action occurs in the intestines. Milk and animal tissues have also been found capable of this reduction. Recently, Oberst and Snyder (56) have studied the action of tissue homogenates on glyceryl, l-glucosan, mannitol, and sodium nitrates. Liver, skeletal muscle, and blood were found to form inorganic nitrite from these organic nitrates. Liver tissue reduced 44 per cent of glyceryl trinitrate in 45 minutes at 37°C. Krantz, Carr, and Forman (45) have observed the rapid disappearance of aryl nitrite from the blood with the formation of nitrate, indicating the tendency toward oxidation of nitrite in the body, rather than the reduction of nitrate. This was confirmed in vitro with alkaline hydrolysis by Snyder, Klahm, and Oberst (68). It appears, therefore, that both reactions may occur in the blood or tissues.

Krantz, Carr, Forman, and Cone (47) showed that erythrityl, glyceryl, and mannitol nitrates lose most of their depressor activity after being subjected to alkaline hydrolysis. Isomannide dinitrate, shown to be only slightly affected by alkali, maintained its full depressor response

after attempted hydrolysis. However, liver homogenate yields a large amount of nitrite by action on isomannide dinitrate (57). This serves to indicate that hydrolysis rates in vitro are not necessarily parallel to action by cellular constituents. Oberst and Snyder found two systems in liver which reduce nitrates, one heat labile and one heat stable. Muscle and blood contain a heat stable component. The optimum pH, concentration of agents and of homogenate were studied (56). These authors hold untenable any hydrolysis to inorganic nitrate and then reduction to inorganic nitrite. They hold as possible the explanation that organic nitrite may be formed with subsequent hydrolysis. They studied the enzymatic reduction of a large number of nitrates, and list them in order of their lability. It is interesting to note that no monohydric alcohol nitrates gave nitrite except monoethanol amine nitrate. The amino group may render lability upon the nitrate group. Snyder, Alahm and Oberst (op. cit.) observed that those nitric esters which yield considerable amounts of nitrite on alkaline hydrolysis contain at least two nitrate groups on adjacent carbon atoms without the interposition of a non-nitrated carbon atom. Those which were relatively resistant to alkaline hydrolysis had an unsubstituted carbon atom between the nitrated carbon atoms. Isomannide dinitrate is in the latter category and thus resistant to alkaline hydrolysis while being quite vulnerable to liver homogenate

as mentioned previously.

These investigators found that glyceryl trinitrate suffered the reduction of 52 per cent of its nitrogen to nitrite in 30 seconds and of 70.6 per cent in two hours. The hydrolysis patterns in alcoholic potassium hydroxide of about twenty-eight other nitrates are also given. Snyder and Oberst (69) have studied the relative oxidation-reduction potentials of about 26 nitrates. Isomannide dinitrate was found to have the highest potential. The isosorbide analogue is second and glyceryl trinitrate is third. Mannitol hexanitrate is thirteenth, although it is more potent than the others named. Thus potency is not a simple function of reduction-oxidation potentials nor of hydrolysis by means of alkali or homogenized tissue.

One point may come to mind after studying the foregoing arguments and data. An organic nitrate may merely touch or enter the cell membranes, including smooth muscle, or penetrate the interior of the cell. What happens in these sites may not be reflected by blood levels of the metabolic products. Any nitrite or further decomposition products formed in the cells may remain there. Blood analysis would thus yield no evidence of their formation.

The exact intracellular fate of organic nitrites and nitrates has therefore not been proved, although from the studies on demolished cells quoted, some insight has been attained.

It is not valid to conclude that the reduction of organic nitrates by naked protoplasm can be applied to intact cells, nor do the workers who demonstrated the ability of intracellular substances in this action suggest that such analogy may be drawn. They have, however, caused us to become aware of the strong reducing nature of agents in the cells, at least as far as organic nitrates are concerned, and have thus introduced a strong potentiality into the concept that nitrite (or other decomposition products) may be the pharmacologically active product of organic nitrates. Perhaps the reduction of these compounds somewhere on or within the muscle cell, or the oxidation of the reducing agents is the necessary event which initiates relaxation. This was Marshall's view fifty years ago. That it is probably not some "static" nature of the nitrate group which causes relaxation is suggested by the inactivity of the nitrate ion.

An interesting question is whether the action of these substances involves a reaction or chain of reactions which are peculiar to smooth muscle cells, or whether such reactions are occurring in all cells to some extent. If, for example, the oxidation of glucose be inhibited at some link, and this interference cause a muscle cell to relax, it would then be probable that glucose oxidation in all cells is depressed. There are of course many reactions common to muscle cells and other organ and tissue cells.

Thus there is the possibility that many organs and tissues suffer depression of their activities during the sojourn of nitrates and nitrites in the body. There is also the possibility that muscular relaxation by nitrate action involves predominantly the contractile mechanism or its specialized source of immediate energy, the high energy phosphate bonds. The interference with breakdown or resynthesis of these energy rich materials could cause weakness and fatigue.

A smooth muscle cell in a state of partial or complete contraction may be maintaining its tone through the mechanism of a complex equilibrium. The energy spent may be considered as being shuffled back and forth among the several systems involved, with the continuous breakdown and resynthesis of all materials involved. If nitrate or nitrite inhibits the system at any point, the contractile mechanism will sooner or later experience the decrease in the energy it had been consuming. Relaxation must then result, and the time required for it will depend upon how near to the direct source of energy for contraction the system is depressed. Thus, if aerobic systems only are depressed, the muscle can continue to contract for a longer period than if the high energy phosphates are held at bay. It should be explained that the chemistry of smooth muscle contraction has not yet been elucidated even to the degree to which has that of skeletal muscle.

We know that skeletal muscle is also weakened and later killed by nitrites. The work of Leech in this regard has been mentioned. This investigation also demonstrated that the frog's heart is weakened, although accelerated, by perfusion with a solution of sodium nitrite. The nitrites may accordingly be considered as depressants of all muscle tissue. The greater sensitivity on the part of smooth muscle is so outstanding, that in therapeutic use, these agents have a negligible effect upon the efficiency of striated muscle.

CHAPTER III

EXPERIMENTAL

AIMS OF PRESENT RESEARCH

The number and type of organic nitrates which have been synthesized and studied pharmacologically is limited. Since the nitrate and nitrite groups confer upon aliphatic hydrocarbons a marked and dramatic action, the sudden relaxation of smooth muscle cells, there is justification for exploring additional types of nitrates in an attempt to develop more useful agents possessing that action. Furthermore, since the mechanism by which these depressor nitrates and nitrites relax the muscle cell is unknown, there may be much to be gained by elucidating the features of their action and factors which modify it. A class of substances which are so potent in altering as important a homeostatic function as smooth muscle tone should be understood as well as possible.

As approach to a greater understanding of nitrate action can take several lines of investigation. The role of oil and water solubility ratios can be further studied in the homologous nitrates of glycollic esters. This work was begun by Krantz, Carr, Forman and Cone (op. cit.), and is described in Chapter IV. Greater duration of action

may be afforded by proper manipulation of molecular configuration and the physical properties associated therewith. This approach will be attempted in the present work. In addition, nitric esters of other hydroxy acids will be studied, e.g., the nitrates of malic and lactic acid, already studied briefly by the above workers, and of tartaric, saccharic, and mucic acids.

Another direction in which work in this field should be advanced is the elucidation of the mechanism by which nitrites and organic nitrates relax smooth muscle. Some experiments are herein reported on the oxygen uptake of living tissue slices in vitro under the influence of sodium nitrite. No previous reports of this nature have been found. The effect of sodium nitrite on the enzyme or enzymes in the muscle fibre which catalyze the hydrolysis of adenosine triphosphate will be studied. Since the latter substance is probably the first source of energy for the contractile process, such effect, if any should be known. The effect of sodium nitrite and organic nitrates upon cytochrome oxidase and cytochrome reductase will be studied.

Ideas, bearing on the subject of nitrate and nitrite action occurring to the author, which are not investigated at this time, will be discussed for possible future work.

METHODS

Dogs have been predominantly used in the blood pressure experiments, with several cats and rabbits. Ether anesthesia has been used throughout with the exception of local anesthesia in two dogs.

Blood pressure was recorded by the use of a mercury manometer, using the carotid or femoral artery. The zero base line was marked at ten second intervals. The height of the pulse tracing was doubled in order to give systolic blood pressure. Diastolic pressure was not recorded, the inertia of the system being too great, but changes in pulse volume could be seen.

Respiratory rate and relative depths were recorded in most experiments above the blood pressure tracing.

Intravenous injections, made by use of cannulae in the saphenous vein, were followed by 1 to 3 cc. of a 0.9 per cent sodium chloride solution, the volume depending upon the size of the animal.

Further details pertinent to specific blood pressure experiments are given in connection with the work involved. The methods used in other phases of the investigations are mentioned or described in the appropriate sections.

CHAPTER IV

PHARMACOLOGY OF NITRIC ESTERS OF ALKYL GLYCOLLATES

Marshall (53) was the first to study nitrated organic acids. He wrote in 1912,

> The nitric esters of tartaric, citric, and lactic acids, neutralized with sodium bicarbonate, produced, when injected intravenously, no fall in blood pressure whatever, and the nitric esters of ethyl-citric and ethyl-lactic acids caused a fall only after a lapse of several minutes.

In 1940, Krantz, Carr, Forman, and Cone (47) showed that another hydroxy acid nitrate is effective as a depressor. They studied the action of the nitrates of sodium, ethyl, propyl, butyl, and heptyl glycollate. The synthesis of these esters was reported in 1941 by Forman, Carr, and Krantz (29). Krantz et al determined for each glycollate nitrate the concentration which would just give a small but definite depressor response (10 mm. Hg) in dogs. They also determined the oil over water coefficient for each. Their results and the formulas of the compounds are shown in Table 18 in the Appendix.

They thus demonstrated that a carboxylated organic nitrate can lower blood pressure. They also showed that alkyl esters of glycollate nitrate are considerably more potent as depressor agents than the sodium salt, and that

the potency of the esters increases as the ester group is lengthened. The relationship between oil over water coefficient to the potency and to the length of the ester group is emphasized by these investigators. The role of oil over water coefficient in the pharmacology of various organic nitrates has been mentioned by many earlier writers, but here, in a homologous series, it can be more easily studied.

A closer investigation of the pharmacology of this homologous series of glycollate nitrates has been attempted in the present work. The series has been extended by the synthesis of additional glycollate nitrate esters by Iwamoto and Harmon (42).

PROPERTIES

Physical Properties

The nitrates of alkyl glycollates are clear liquids at least up to and including the decyl derivative. They are colorless or pale straw colored, possess fruity odors and a burning taste. They are sparingly soluble in water with the exception of the methyl compound which dissolves to the extent of 2.4 per cent. Table 3 lists the actual solubility of each member of the series. They are quite soluble in alcohol.

Chemical Properties

The saturated aqueous solutions of these nitrates

possess very constant degrees of acidity. This is not due to nitrous acid, but apparently to replaceable hydrogen on the alpha carbon. Sodium derivatives may thus be prepared. These are white solids, stable, and very soluble in water. In describing their pharmacology (Chapter V) these sodium derivatives or salts will be variably called neutralized alkyl glycollate nitrates, ester salts, and by specific names containing the actual alkyl ester present, e.g., sodium isobutyl glycollate nitrate. The Appendix contains the type formula for these ester salts (Table 19).

PHARMACODYNAMICS

Depressor Action of Alkyl Glycollate Nitrates

All homologs in this series so far tested have produced falls in blood pressure which are qualitatively quite similar. Several features which aid in an understanding of nitrate action may be demonstrated by using the ethyl compound as an example. The effects of this substance upon blood pressure when given both by vein and by the intestinal route are reported. A typical record of the blood pressure and respiratory tracing is shown in Figure 1.

Results

The results of intravenous injection into two dogs, Nos. 26 and 27 have been summarized in Table 1 and Figure 2, Four different doses were studied in an attempt to discern the various responses possible, especially the duration of

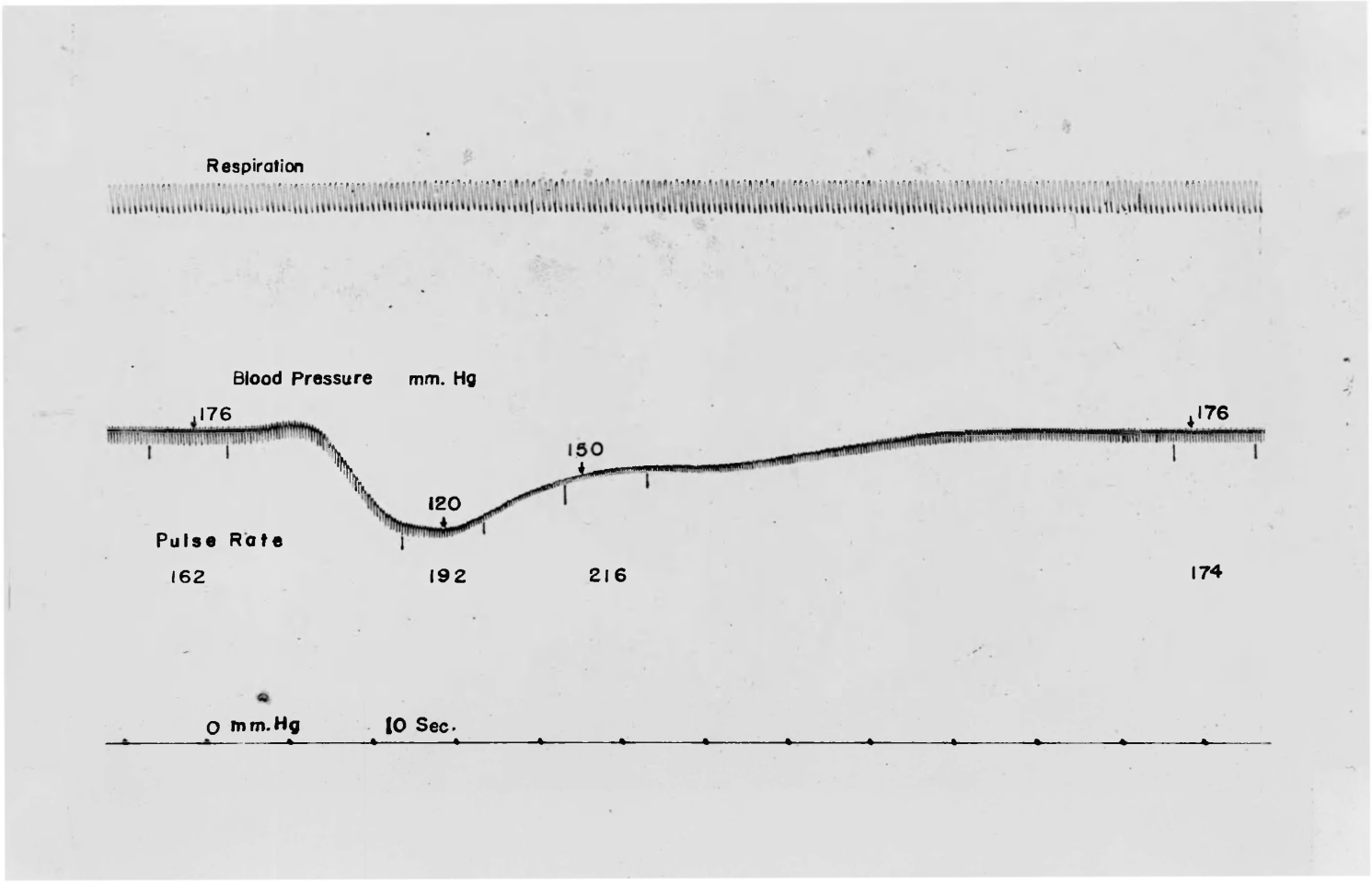

FIG. I. RESPONSES OF RESPIRATION, BLOOD PRESSURE, AND PULSE RATE IN DOG NO. 27 TO ETHYL GLYCOLLATE NITRATE

Ether anesthesia. Carotid artery blood pressure. Dose: 5.2 mg. per kg. intravenously. Pulse was counted for 10 seconds at places shown; after conversion to rates per minute, figures are shown beneath tracings of pressure. Blood pressure at arrows is indicated above the tracing. Injection approximately coincided with first 10 second interval shown.

TABLE 1

Depressor action of the nitric ester of ethyl glycollate

Dog No.	Dose of nitrate	Blood Pressure			35 second level per cent	Time for 99% recovery
		Initial	Minimum reached			
	Mg./kg.	Mm. Hg	Mm. Hg.	Per cent		Minutes
26	1	140	124	89	93	9
		142	122	86	97	4
27		172	140	81	87	3
		178	146	82	93	1.5
	Mean			85	93	
26	5	136	92	68	91	8
		144*	100	76	81	8 (92%)
		130*	98	75	84	4
27		176	120	68	82	1.5
	Mean			72	87	
26	10	144	100	69	76	2.7
27	38	172	80	47	68	1.7
	(in 50% alcohol)	165	66	40	45	**
	Mean			44	57	

Intravenous injections. Ether anesthesia. Carotid artery pressure recorded. *See text for comment. ** Respirations ceased temporarily, revived; blood pressure recovered 90 per cent. Above data are summarized in Figure 2.

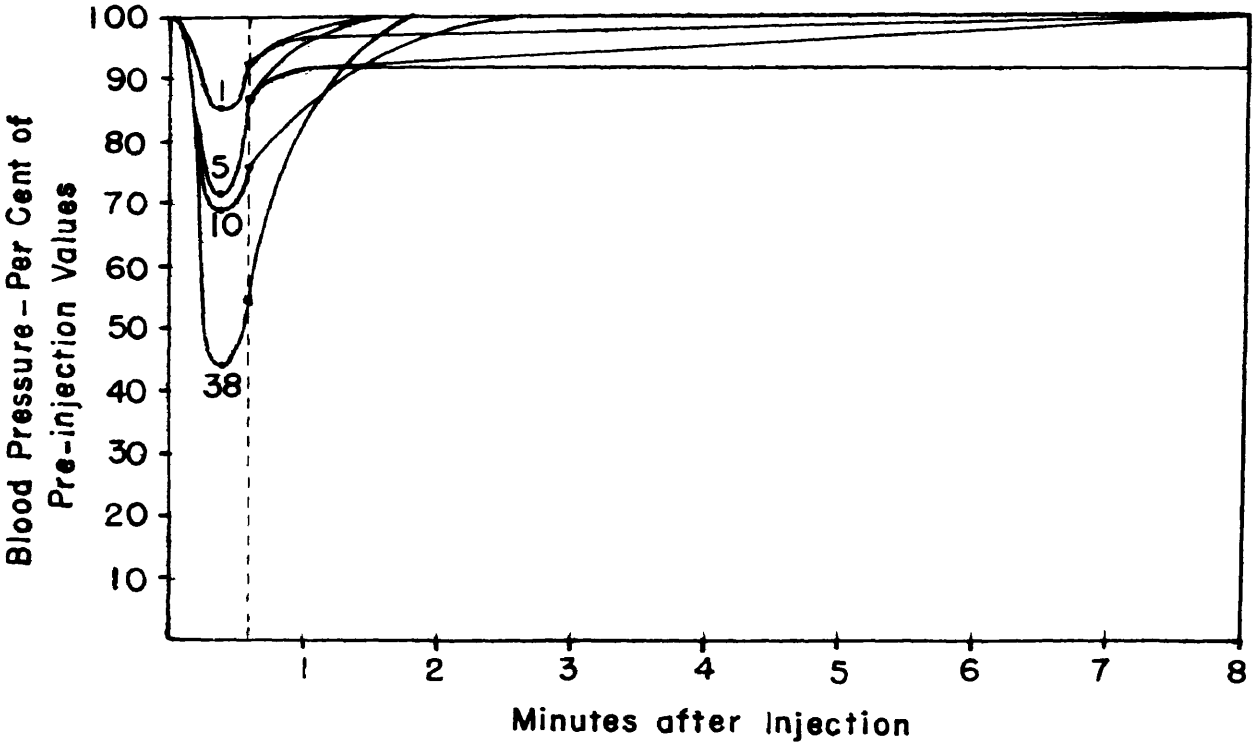

FIG. 8. DEPRESSOR ACTIVITY OF ETHYL GLYCOLLATE NITRATE
DATA FROM TABLE I

Dose for each group indicated, mg. per kg. The curves represent mean responses up to the 35 second time ordinate. Beyond the latter, the extremes of responses to each dose are sketched.

depressor action. In each dog, the smaller doses were given first, increasing in order, with two exception which are marked with asterisks in the table. These two injections were given after one injection of the next larger dose, 10.5 mg. per Kg. Similar doses are grouped together for convenience of comparison and of computing averages of the figures. The figure shows the results somewhat schematically. In the figure, the blood pressure at the time any injection is made is shown as 100 per cent and all falls are shown as percentage of that value. The table shows actual pre-injection levels for each dose, and since these are, in each animal, fairly constant, it is apparent that the condition of the animals was not noticeably deteriorating during the experiments.

The pressure at 35 seconds after the injections is shown, also as per cent of pre-injection values. This time was selected because there was usually a slight decrease in the recovery rate after that point. Finally, the time at which recovery was 99 per cent of previous normal level was recorded. The figure shows all these results in two phases. The responses to a given dose up to the 35 second point are shown for simplicity, as the mean of the several injections. The figures making up each mean are reasonably similar. The responses after the 35 second point are shown individually, because the variability is greater. True events are more clearly depicted in that manner.

In the group of injections in which the dose was 1 mg. per Kg., the solution was 0.01 molar, or 0.15 per cent in 0.85 per cent sodium chloride solution. The other doses and solutions were as indicated. The largest dose was of necessity given in 50 per cent ethyl alcohol.

Discussion

It is possible that precipitation of droplets of the ester in the blood occurred as the injection was made. If solution of these particles recurred, a more prolonged depressor effect might be expected. No such response was observed in this one injection. Not listed or drawn was a final injection of a still greater dose, 60 mg. per Kg, in 75 per cent alcohol also with rapid recovery.

Prolongation of the response by increasing the dose of this ester is apparently not possible. The falls in pressure are immediate, depend for their magnitude on the size of the dose, and are quickly abated. No great tolerance is manifest.

On one occasion, a 10 per cent solution of ethyl alcohol, saturated with the butyl ester of glycollic acid nitrate, was shaken with mineral oil and the aqueous layer then injected into a dog (No. 22). The solution had completely lost its depressor effect by having its vasodilator agent extracted by the oil. The original solution had lowered the pressure of the same animal 12 per cent.

Effect of a Sympathetic Nervous System Blocking Agent on the Duration of Depressor Action of Alkyl Glycollate Nitrate

An effort to afford a more prolonged response to one of these esters was made by administering the butyl compound to a dog (No. 21) which had previously received Dibenamine, 20 mg. per Kg., as a sympathetic nervous system blocking agent. Both compounds were given intravenously.

The fall obtained was fleeting with complete recovery in 40 seconds. The fall was from 106 mm. to 84 mm. Hg. Another dog (No. 22) showed rapid recovery with Dibenamine premedication, when the blood pressure was only 68 mm. Hg at the time the nitrate was given.

In these two animals it appeared not to be possible to depress the blood pressure for a longer period by the use of an agent which prevents adequate sympathetic motor outflow. The smooth muscle fibres which were relaxed by the nitrate regain their tone as quickly without vasomotor reflex integrity as with it.

This fact prompted a few experiments to determine if possible the major site of action of injected nitrates such as those of alkyl glycollates. Two dogs were used. The brachial artery was tied distal to a cannula pointing toward the aorta. Alternate injections of equal doses of one of these nitrates were made into this artery and into the saphenous vein. The injections were followed by 3 cc. isotonic sodium chloride solution. Falls in pressure

followed in 10 to 11 seconds after both arterial and venous injections. In one dog, two intraarterial injections gave falls of 10 and 13 mm. Hg, and two intravenous injections gave falls of 11 and 13 mm. In another dog, the same time periods for responses were observed, but the depths of the falls differed.

These observations suggested that after intravenous injections, the pulmonary vascular tree may be the area of greatest dilatation, if not exclusively so. It is difficult to believe that dilatation would not be greater in the lungs than any where else, because of a greater concentration in that relatively small space. On the other hand, weakening of the contractions of the heart might contribute to the fall, after the drug has passed through the lungs and into the coronary circulation. This action of the nitrites has already been mentioned.

When injections were made into the arterial system, retrograde via the brachial artery, the falls in pressure began after the same interval of time as after injections into the vein. The arteries, arterioles, capillaries and veins have all been shown to be relaxed by nitrites or nitrates. The quantity injected is distributed to a greater mass of tissue when injected intraarterially, and the concentration per unit weight of tissue will be much less than that in the lungs after intravenous injections. When large doses are given intravenously, both circulatory beds may

be relaxed, and longer action seen.

Depressor Activity After Injection Into the Gut

Into the intestine of Dog No. 28 was injected 0.5 ml. of 0.9 per cent sodium chloride solution. The blood pressure fell from 100 to 96 mm. Hg at which level it remained for 5 minutes. A loop of gut was again picked up and the needle inserted preparatory to the next injection. The pressure fell to 90 and remained there. Then 0.5 ml. of pure ethyl glycollate nitrate (0.19 Gm. per Kg.) was injected. The blood pressure began declining after 30 seconds, and in 90 seconds was 78 mm. It slowly declined to 50 by the end of an hour. The animal was then sacrificed.

The injection of physiological salt solution serves as a control for the second injection in which the dose was 5 times greater than the highest shown in Table 1. The marked prolonged hypotension obtained appears to have been caused by the drug.

Other Effects of Alkyl Glycollate Nitrates

The heart rate is accelerated during and immediately following the depressor action of these esters administered intravenously. The greatest effect comes usually during the immediate recovery period when the rate may be increased by a third. Thereafter there is a gradual decrease in rate, and, in some animals the rate is actually depressed for a few minutes. Figure 1 shows such changes.

The pulse volume is decreased at the depth of the fall and for varying periods thereafter. Figure 1 shows this effect to be maximal on the ascending limb of the record, when the rate is greatest.

Electrocardiograms taken on 4 days before and after intravenous injections of these nitrates showed no abnormality during or after the effect other than the increase in rate.

Respiration is unaffected by the usual experimental depressor doses, but excessive amounts cause depression of rate and depth for short periods. In such cases, deep depressor effects are in progress.

BIOASSAYS

This series of compounds, whose members differ only in the number and arrangement of carbon atoms in the alkyl ester group, presents an opportunity for the study of the effects of several factors on the depressor activity elicited by the molecule. The molecular weight may be increased as the ester chain is lengthened. Water solubility and oil over water coefficients will vary as will many other physical characteristics. It may be determined which characteristics are primarily responsible for variations in potency among members of the group.

Method

A method of bioassay was devised similar in principle to that of Epinephrine Solution official in the United

States Pharmacopoeia except that falls in blood pressure are measured instead of elevations. Dogs under ether anesthesia were used, with recording of femoral artery blood pressure. Solutions of all members of the series from the propyl ester to the octyl glycollate nitrate were made at 0.010 molar concentration (weight to volume). The solvent was the lowest concentration of ethyl alcohol in distilled water which would hold the compounds in solution at room temperature. Such requisites of alcohol strength were not exact, but probably within a few per cent. Table 20 in the Appendix shows the actual concentrations of alcohol used for the various compounds. The effect of the alcohol was controlled by injecting various concentrations without the nitrate. Since the volume of each injection was small, the effect of alcohol on the blood pressure was in no case great enough to influence the assay.

Early trials revealed the isobutyl ester of glycollate nitrate to be one of the most potent of the entire group. It was decided to compare each of the other homologues with it as a standard. Accordingly, weaker solutions of the isobutyl compound was prepared, e.g., 0.009, 0.008, 0.007, and down to 0.001 molar concentrations, by diluting the 0.010 molar solution with appropriate amounts of water. This reduced the alcoholic strength at the same time as indicated in the table. Using the dogs blood pressure as a test object, 0.01 M. solutions of all other glycollate nitrate esters were matched against the appropriate isobutyl

ester solutions.

The actual procedure was the alternate injections of one of the isobutyl solutions and the 0.01 M solution of the ester being assayed. Several injections of each, standard and unknown, were thus made in alternation. The falls in pressure produced by the injections were measured and expressed as per cent of original pre-injection blood pressure. The average of the results obtained with the standard was compared with that obtained with the unknown. If there were no significant difference between the averages, the solutions used were considered to be of indistinguishable strength. When the homologues had thus been matched against appropriate strengths of the isobutyl compound, the actual molarity of the latter was an expression of the relative potency of the members of the series. This can also be expressed as a ratio between the molarity of the standard and that of the unknown. This is called the "isobutyl rating."

In all injections used in assays, the volume used was adjusted so that submaximal responses were obtained. The assays described were successfully accomplished after a number of dogs had been used in developing the most satisfactory technique. Exploratory comparisons are not reported. The development of tolerance was evident during the assays of the n-amyl and n-hexyl esters. It affected the responses of both solutions being compared, as can be seen in their respective tables. The interval between injections was

approximately the same for a given assay, and usually varied from 5 to 8 minutes among the various assays. Shorter intervals were found to result in inconsistent results, and longer ones prolonged the assay so much as to prevent comparison of injections at the beginning with those at the end. A somewhat greater or smaller concentration of the standard or of the unknown was given at the beginning or the end of an assay in order to demonstrate the validity of the comparisons in the assay. Thus a significantly different fall in pressure upon altering the dose attests the sensitivity of the method.

Results

The results of the assays are tabulated in the Appendix in Tables 21 to 29. Table 2 presents a summary of the assays.

The oil over water coefficients of these compounds may be the most outstanding property which determines their relative potency. They are so greatly soluble in oils that a convenient and more accurate method of representing the relative solubilities is to use water solubility alone. This was measured by Iwamoto and Harmon (loc. cit.) by determining the density of saturated solutions of the esters in distilled water and computing the solubility. Table 3 shows the figures thus obtained.

TABLE 2

Summary of assays of alkyl glycollate nitrates

Glycollate nitrate ester - 0.01M.	Equivalent molarity of isobutyl homolog	Isobutyl rating (Relative potency)
Methyl		
Ethyl		
Isopropyl	0.004	0.4
n-Propyl	0.004	0.4
(Isobutyl)	(0.010)	(1.0)
sec-Butyl	0.005	0.3
n-Butyl	0.004	0.4
Isoamyl	0.010	1.0
n-Amyl	0.006 to 0.007	0.65
n-Hexyl	0.006	0.6
n-Heptyl	0.010	1.0
n-Octyl	0.006 to 0.007	0.65
n-Nonyl		
n-Decyl		

TABLE 3

Solubility in water of alkyl glycollate nitrates
Grams per 100 ml. at 30°C.

Alkyl Ester	Normal	Iso	Secondary
Methyl	2.444		
Ethyl	0.291		
Propyl	0.181	0.176	
Butyl	0.135	0.131	0.118
Amyl	0.099	0.092	
Hexyl	0.124		
Heptyl	0.095		
Octyl	0.103		
Nonyl	0.111		
Decyl	0.115		

From the table it will be seen that the solubility of the substances decreases from the methyl to the amyl members, with irregular changes among the higher homologues. The branched chain compounds are less soluble than their respective normal isomers. Isoamyl and n-heptyl esters are the least soluble of the entire group.

The possibility that the esters may precipitate in the plasma after injection was entertained and a brief experiment devised. The solutions used for assays had a nearly critical concentration of alcohol for solubilizing the esters. It was found that the members having four carbon atoms or less in the ester group could be diluted with water without precipitation. The n-amyl compound was easily precipitated, but the precipitate redissolved in excess water. The isoamyl and the higher members were easily precipitated by addition of water and could not be redissolved by further dilution

with moderate amounts of water. Plasma might conceivably dissolve the higher homologues to a greater degree than does water. Any precipitation which occurs in the plasma might result in the suspension of very small particles, which would eventually dissolve as admixture of blood continues.

The relationship between the solubility curve and that of the relative potency of most of the compounds is apparent in Figure 3. The curve representing relative depressor potency is based upon the "isobutyl ratings". As the solubility decreases the potency increases. The heptyl compound is the least soluble and the most potent of the straight chain esters.

A comparison was made between a month old solution that had been used repeatedly, and a freshly made identical solution. One of the more volatile members, n-propyl, was selected. Table 4 shows their relative strengths in reducing blood pressure, again by alternating the injections. Both the new and the old n-propyl glycollate nitrate solutions were 0.010 M. in 5 per cent alcohol. The results reveal that there is no significant difference between the two series. In addition, two injections were made of a fresh solution of the same nitrate, in 52 per cent alcohol. The average was not different from that of the other two means. This suggests that the high concentration of alcohol has no influence upon the effects of the drug. In the assays,

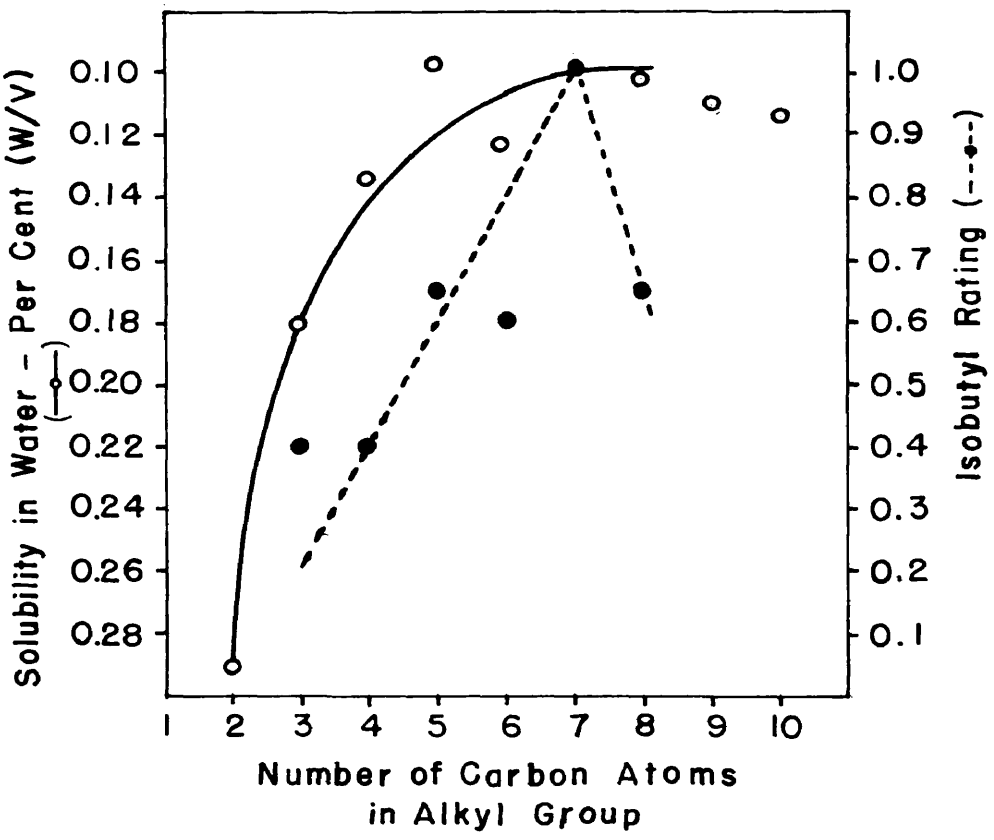

FIG. 3. RELATIONSHIP BETWEEN POTENCY AND WATER SOLUBILITY AMONG NORMAL CHAIN ALKYL GLYCOLLATE NITRATES

TABLE 4

Comparison of old and fresh solutions of
0.01 molar n-propyl glycollate nitrate
Dog No. 66 0.25 cc per kg.

Per cent falls in blood pressure		
Old solution in 5% alcohol	New solution in 5% alcohol	New solution in 50% alcohol
7.9	9.2	7.9
9.3	8.7	8.0
7.3	8.0	
6.7	8.0	
7.3	7.3	
Mean 7.7	8.2	8.0

Initial blood pressure for all injections was from 150 to 155 mm. Hg.

control injections of the alcoholic vehicle employed were made, the volume being the same as that of the drug. If the alcohol was found to affect the assay, the experiment was discarded.

Discussion

In establishing what seemed to be the optimum details for a method of bioassaying such compounds as the glycollates, several problems were considered. The concentration of the solutions had to be such as to require as little alcohol as possible for keeping the esters in solution. The concentration of the esters should not be so low that large quantities would have to be injected. In this case the animal could possibly be hyper hydrated during the assay. The strength of esters (0.01M.) used was decided upon after trials of other strengths.

The method of alternating injections of a standard and of a product to be assayed, adjusting their concentration so that nearly equal responses are obtained, is used in many types of assay work. It is to be preferred to the comparison of different degrees of response when that response is a complex biological one.

It is difficult to treat the results with statistical methods. Each animal must constitute an assay. Moderate differences in potency can be detected, but to determine the sensitivity more closely than has been done in these

assays would seem impractical. The drugs must act in the presence of vasomotor and cardiac reflexes. The former should not be abolished because it is desirable to have complete recovery following each injection. In this way, all blood pressure falls begin from nearly the same level. They are therefore more comparable than if the reflexes did not restore that level after the injections.

Using the same technique of assaying, a 0.01 M. octyl glycollate nitrate solution was compared with a 0.002 M. mannitol hexanitrate solution. Four injections of each were made. The results gave mean values of 21.8 and 22.4 (per cent fall in blood pressure), with standard deviations of 2.0 and 2.1, and standard errors of 1.0 and 1.1 respectively. It was determined that no significant difference existed between the two means.

From the ratio of the molarities, mannitol hexanitrate is seen to be 50 times more potent than the octyl compound. It is much less soluble in alcohol and in water.

TOXICOLOGY

Because of the acid reaction of their aqueous solutions, the alkyl glycollate nitrates may be expected to damage tissue. Small doses by mouth or by vein may be adequately buffered. No data were sought on this question.

Rats tolerated intraperitoneal injections of 87 mg. per kg. of the ethyl compound in saturated aqueous solution

without observable changes in behavior or appearance. The minimum lethal intraperitoneal dose was found to be 0.8 grams of the undiluted ethyl ester per kilogram (rat). Methemoglobinemia was marked, even in survivers of such doses, for more than 6 hours. Respiration was depressed and marked weakness became evident. After lethal doses, convulsions preceded death in most animals.

Darkening of the color of the blood or mucous membranes, during repeated injections in dogs was never seen. More than 30 injections were given during the assays to several dogs, totaling not more than 100 mg. per kg. in two or three hours.

CHAPTER V

PHARMACOLOGY OF THE SODIUM SALTS OF ALKYL GLYCOLLATES

DEPRESSOR ACTIVITY

The formation of the sodium derivatives of the alkyl glycollate nitrates was mentioned in Chapter IV. The acidity of the unneutralized esters, their reaction with alkali, and the great increase in solubility upon neutralization are the chief factors to be emphasized. The formation of a cyclic compound by this treatment is reasonably well substantiated by the work of Iwamoto and Harmon (op. cit.).

The variability of depressor potency among the unneutralized esters was established by assays described in Chapter IV. Neutralization appears to nullify the varying influence of the alkyl group upon the potency of the resulting compounds.

Method

The sodium salts of several of the alkyl glycollate nitrates were prepared in 0.05 M. solutions in water. Injections were made intravenously in doses of 0.75 cc. per kg. Fourteen dogs, two cats, and one rabbit were used. One dog, No. 36, received local anesthesia only. All other

animals were etherized. One dog, no. 57, was anesthetized, then pithed before the nitrate was given.

Results

Table 5 shows the depressor responses to these injections as per cent of original blood pressure at the point of maxium effect, and in most animals at 15 minutes after the injections. The maximum effect was usually produced within one minute.

Examination of the table reveals that the maximum depression of blood pressure for dogs is similar in degree to that of the other animals. There is reasonable constancy in the responses. It will be noticed that the pressure fifteen minutes after injection is still considerably depressed. In most animals there is a partial recovery by the end of thirty minutes.

Subsequent injections give variable responses. Most animals show a step - like lowering of pressure on repeated injections until shock levels are reached. Methemoglobinemia is produced by all injections, as will be described.

Several dogs were given sodium nitrite in order to compare the responses with those seen with the salts of the alkyl glycollate nitrates. They were qualitatively similar, e.g., duration of depressor effect. Quantitative comparisons of potency were not made with sufficient accuracy to

TABLE 5

Maximum depressor effect of equimolecular amounts of the sodium salts of several alkyl glycollate nitrates.

Alkyl Ester	Dose mg./kg.	Dog No.	Per cent of initial blood pressure at maximum effect		15 minutes after injection
Ethyl	6.5	29	78.0		79.7
		32	74.5		
		Average		76.3	
n-Propyl	7.0	34	82.0		
		35	69.6		82.1
		36*	76.5		82.5
		37	62.7		62.7
		Average		72.7	
	(Rabbit)	5	74.0	74.0	74.0
Isopropyl	7.0	50	79.9		
		51	81.8		
		52	74.7		79.0
		53	80.0		
		Average		79.1	
n-Butyl	7.6	38	85.3		91.3
		58	64.9		72.0
		Average		74.1	
		57**	72.2		
	(Cat)	5	62.5		
	(Cat)	6	74.5		
		Average of Cats		68.5	
Isobutyl	7.6	44	74.5	74.5	
Grand Averages of dogs (Omitting pithed dog No. 57)			75.6 ± S.D. 6.5		78.5 ± S.D. 9.0

Intravenous injections. Ether anesthesia with exception: *Dog 36 received local anesthesia. 0.05 M. solutions used in each case. **Dog 57 was pithed before injection.

permit a conclusive opinion. Inorganic nitrite did seem roughly equivalent in potency, on a molar basis, to the ester salts. Suspicion was aroused that the latter may readily hydrolyze to give nitrite ions.

In addition to the salts listed in the table, those of the heptyl and nonyl glycollate nitrates were also prepared and found to give prolonged falls of blood pressure.

The absorption and depressor action of the salts injected into an exposed loop of small intestine was demonstrated six times in two dogs. With the same dose as that used intravenously (Table 5), a 19 per cent fall in pressure was produced. The fall began within 5 minutes, was maximal in 12, and recovered in 36 minutes. A second dose was somewhat less effective (11 per cent fall). Doses twice the foregoing ones gave falls ranging up to 33 per cent of normal pressure and lasting up to one hour.

Discussion

A comparison of the potency of the sodium derivatives of the alkyl glycollate nitrates to that of the unneutralized esters may be made. The sodium isobutyl compound, as shown in Table 5, gave a reduction in blood pressure to 74.5 per cent of the initial level. The dose used was 0.75 cc. per kg. of a 0.05 M. solution, or 7.6 mg. per kg. Approximately the same immediate responses were obtained with the unneutralized isobutyl ester in the assay of heptyl glycollate

nitrate (see Table 28, Appendix). In these injections the dose was 0.13 cc. per kg. of a 0.01 M. solution, or 0.21 mg. per kg. The latter dose, compared to the former gives a ratio 0.21: 7.6, or 1:36. This suggests that in forming the sodium salt, the original nitrate ester, sparingly soluble in water, is greatly weakened. The resulting soluble salt can be given in much greater dose and a greatly prolonged depressor effect ensues. Methemoglobin formation now becomes evident.

The action of one of the salts in the pithed animal confirms the peripheral action of the nitrate. As long ago proved for other nitrates and nitrites, the brain plays a very minor role in nitrate action.

NITRITE CONTENT OF THE PLASMA

The similarity between blood pressure responses to the sodium derivatives of the alkyl glycollate nitrates and those to sodium nitrite prompted the investigation of inorganic nitrite blood levels after injection of one of the ester salts.

Method

A quantitative test for inorganic nitrite was made upon plasma drawn at varying intervals after intravenous injections of the nitrates in three dogs, and of sodium nitrite in one. The method used was that of Ilosvay as modified by Rath (62) Equimolecular amounts of all agents were

administered. Arterial and venous blood samples were repeatedly analyzed as early as the first minute and as late as two hours following injections. Definite and prolonged depressor effects were produced by each drug.

Results

Normal nitrite values in all animals were 10 or less gamma per 100 cc. blood. An increase to 26 gamma per cent was observed in venous (jugular) blood one minute after the injection of a depressor dose of sodium nitrite (2.6 mg. per Kg.). No such increase was seen in venous blood after the injection of sodium isobutyl glycollate nitrate, nor of its isopropyl homolog. Analyses for the latter two were first made two minutes after injection. Arterial blood contains a much larger amount of an injected drug such as sodium nitrite, as was realized by Rath (op. cit.). Nitrite content in arterial blood was moderately elevated by the above injections for a few minutes only.

Larger doses of sodium nitrite were shown by Rath to produce arterial blood levels as high as 1110 gamma per cent, using six times the dose employed in the foregoing experiment. As stated previously, the level is quickly reduced. Rath did not report venous blood concentration of sodium nitrite. Using his higher dose, the present author found juglar venous blood to contain 210 gamma per cent nitrite 2 minutes after the injection. By the end of 30

minutes only about 10 gamma per cent remained. During that time, the arterial blood levels of nitrite were at least four times higher than the venous blood values. This might mean that peripheral tissues are metabolizing most of the nitrite that arterial blood brings to them. The lungs, of course, first receive the injected dose. These organs may retain some of the nitrite in their interstitial fluid and slowly release it back into the blood by simple diffusion. The phenomenon of higher arterial blood nitrite content than venous content was definite in four dogs.

Discussion

All that can be said concerning the liberation of nitrite from the ester salts is that the small number of observations herin reported are compatible with such conversion. If that conversion occurs, it is probably dependent only upon contact with water, and not necessarily upon contact with blood elements. The salt of an alkyl glycollate nitrate responds to some of the tests for nitrite *in vitro*. Many other organic nitrates respond similarly. Further explanation of the action of this sodium derivative must await more chemical data.

TOXICOLOGY

After the treatment of these esters with alkali, they become very soluble. They are well tolerated by animals in

doses which were used to demonstrate depressor activity. Rats recover from doses which cause deep cyanosis (methemoglobinemia) and weakness. The minimum lethal dose is greater than 0.5 gram. per kg. for rats, given by stomach tube.

Methemoglobin Formation

Animals which received repeated or large doses of the sodium salts of the alkyl glycollate nitrates showed cyanosis and a brown coloration of shed blood. This change was reversible. A simple test *in vitro* revealed this type of nitrate to be approximately as potent and as rapid in producing methemoglobin as sodium nitrite. Both substances act instantaneously on hemolyzed blood but more slowly on intact corpuscles, as would be expected.

Method

Methemoglobin formation after injection of sodium nitrite and of sodium butyl glycollate nitrate was studied. Each compound was studied in one dog. The drugs were given in 0.05 M. concentration, 0.75 cc. per kg. The doses thus contained the same amount of nitrogen. The method employed for determination of methemoglobin was that of Evelyn and Mallory (23)

Results

In the two dogs, blood drawn 11 and 13 minutes after the injections gave methemoglobin values of 0.62 and 0.64

Gm. per 100 cc. blood, respectively. At the end of 70 minutes the latter value was found to have decreased to 0.25 Gm. The method of analysis is said to be sensitive only as low as 0.20 grams per cent.

Discussion

Further evidence is offered showing that the sodium salts of alkyl glycollate nitrates behave *in vivo* like sodium nitrite. Equimolar doses of the two substances formed nearly identical amounts of methemoglobin upon intravenous injection. The altered pigment produced by the organic nitrate disappeared within 70 minutes after the injection. According to Van Slyke, Hiller, Weisiger, and Cruz (71) normal blood contains methemoglobin to the extent of 0.4 per cent of the total pigment. On that basis, the normal for the two dogs would be 0.60 and 0.43 Gm. per 100 cc., respectively. One dog had a low total hemoglobin level of 10.8 Gm. per cent. The doses used did not produce much methemoglobin. Cox and Wendel (14) gave twelve times the dose of sodium nitrite herein described, or 30 mg. per kg., and found 65 per cent of the blood pigment converted to methemoglobin.

The reduction of methemoglobin, produced by nitrite, to active hemoglobin proceeds at a fixed rate, as shown by Cox and Wendel. They measured the rate of disappearance and found it to be 11.3 per cent of the total pigment (methemoglobin plus hemoglobin) per hour. A standard deviation

of 2.0 was reported.

THEOPHYLLINE COMBINATIONS

Theophylline is the xanthine compound of choice in the routine therapeutic dilatation of coronary arteries. It is sparingly soluble in water, and is usually mixed with an alkaline salt such as sodium acetate; or with ethylene diamine. The mixtures greatly increase the solubility of theophylline.

The possibility of using the sodium salt of an alkyl glycollate nitrate as solubilizing agent presented itself. Accordingly, a mixture of theophylline and one of the foregoing nitrates were prepared and solutions containing one per cent of each were injected intravenously into dogs.

Results

Table 6 shows the depth of blood pressure depression at varying intervals following injections of 0.5 cc of the solution per kilogram. The pressure was lowest two minuted after the injection and thereafter rose very slowly. Definite depression still exists after 12 minutes. Some animals recover thereafter, and some maintain the level of partial recovery. The n-butyl and n-hexyl homologues were also found to be active.

The depressor action of theophylline alone was studied under the same conditions with the exception that glycine was added to render the alkaloid more soluble. The im-

TABLE 6

Depressor activity of theophylline with sodium isobutyl glycollate nitrate

Dog No.	Depression of blood pressure as per cent of initial				
	Time after injection - minutes				
	2	6	12	20	30
90	81.6	83.0	87.5	90.5	90.5
91	71.6	76.9	84.2		112
92	88.0	88.8	89.5	97.8	97
96	83.2	86.2	92.4		
97	75.3	85.2	82.1	78.6	
Mean	80.0	84.0	87.1		
S. D.	6.5	4.9	4.1		

mediate depressor effects upon intravenous injection were quite comparable to those produced by the combination with the nitrate. Recovery was rapid after theophylline alone, requiring only two or three minutes.

More than 40 injections of the combination product were made in eleven dogs in an attempt to evaluate various methods of preparation and relative potency compared with other theophylline products. The results tabulated represent all of the injections made with one particular yield of the material.

As usual for the nitrates and nitrites, this product also caused a variable increase in the ventricular rate. An electrocardiogram two minutes after an injection revealed no change other than an increase in rate from 200 to 240 beats per minute.

Discussion

Theophylline alone produces distinct but transient depression of arterial blood pressure. A sodium alkyl glycollate nitrate solution alone gives an immediate but prolonged depressor response. Their combination in a single preparation acts in the same manner as the nitrate alone. It is probable that only a long series of carefully controlled experiments would reveal whether the alkaloid potentiates the action of this nitrate.

CHAPTER VI

PHARMACOLOGY OF THE NITRIC ESTER OF SODIUM GLYCOLLATE

Sodium glycollate nitrate is a white solid, very soluble in water, and relatively quite stable. It has a bitter salty taste.

PHARMACODYNAMICS

Depressor Action of Sodium Glycollate Nitrate as Initial Intravenous Injections

In their paper, Krantz, Carr, Forman, and Cone (op. cit.) reported:

> A striking characteristic was observed with injections of the nitrate of sodium glycollate. High molar concentrations (0.25 to 0.5) when injected into the dog as the first medication produced a marked and prolonged depressor action. When the pressure returned to normal, a second dose elicited no significant response. The tolerance, however, was confined to the water-soluble nitrates as in animals where this tolerance existed, the water insoluble compounds elicited marked depressor response.

This phenomenon has been studied in the present work. Because of the resistance to a second dose of this nitrate, the primary and secondary injections must be studied separately.

Results. Table 7 and Figure 4 show the results in dogs, cats, and rabbits of the initial dose, 54 mg. per kg., of the drug. Four dogs, Nos. 1,2, 8, 117, of both sexes, weighing from 5.5 to 10.4 Kg. were anesthetized with ether.

TABLE 7

Depressor effects of the nitrate of sodium glycollate. Initial intravenous injections

Animal	Blood Pressure				
	Initial	Minimum reached		Immediate recovery	8 minutes after injection
	mm. Hg	mm. Hg	Per cent	Per cent of initial	Per cent of initial
Dogs					
1	138	100	72.5	85.6	85.4
2	162	120	74.1	97.5	92.6
8	144	112	77.8	110	85.0
117	126	90	71.4	92.0	82.5
Mean			74.0	96.5	85.9
Standard Deviation(S.D.)			2.8	10.4	4.6
3 (*)	104	70	67.3	100	92.3
10 (**)	86	54	62.8	74.5	72.1
Cats					
1	132	84	63.6	89.4	80.0
5	116	84	72.4	100	77.5
Mean			68.0	94.7	78.8
Rabbits					
1	120	74	61.7	None	61.7
2	106	70	66.0	None	66.0
Mean			63.9		63.0

Ether anesthesia and the carotid artery were used with the exception (*) of dog No. 3 in which local anesthesia (procaine) and the femoral artery was employed. The dose in all cases was 54 mg. (in 0.75 cc.) per kg. ** Dog No 10 had signs of "Distemper".

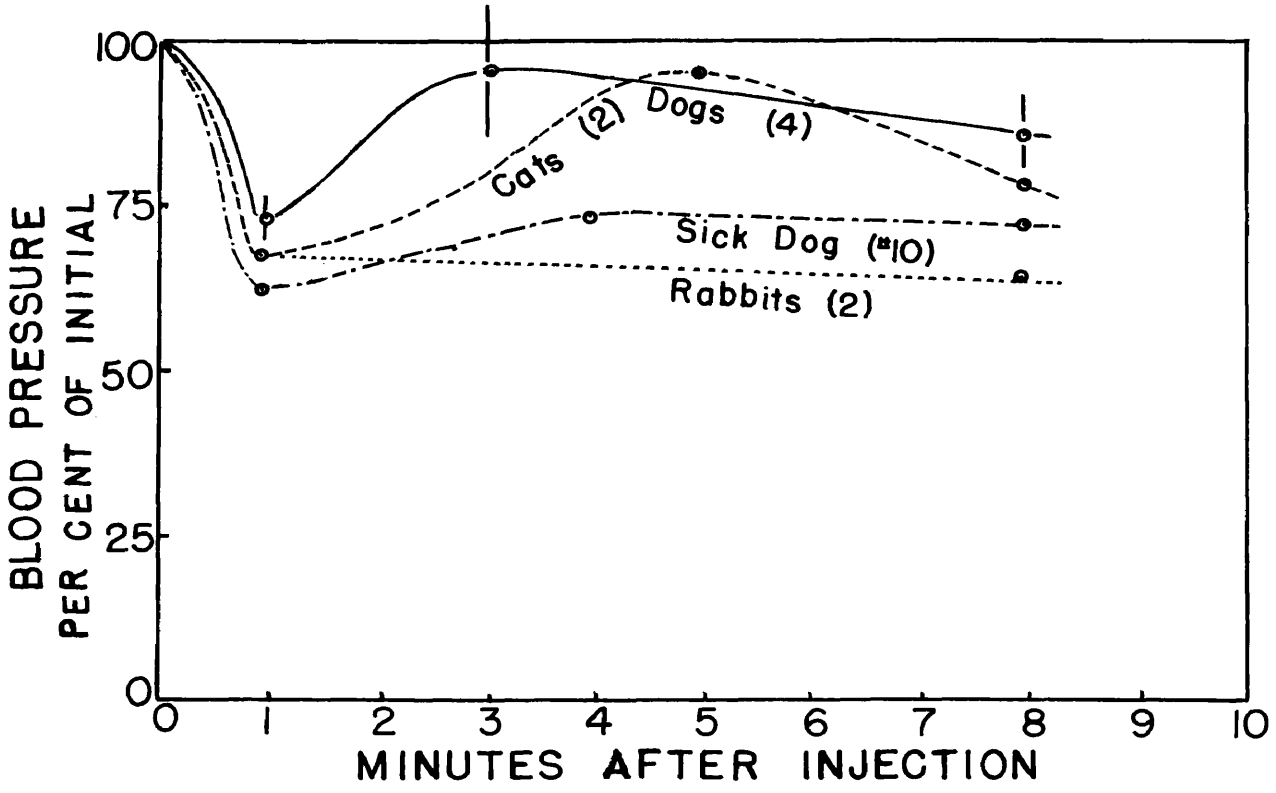

FIG. 4. GRAPHIC REPRESENTATION OF RESULTS GIVEN IN TABLE 7

Points plotted are mean values shown in the table. Curves are sketched to show approximate contours. Standard deviations are shown as vertical lines on one curve. The timing of the events shown was not included in the table.

Initial carotid blood pressure, recorded as described under Methods, varied among the animals from 126 to 162 mm. Hg. Starting from 10 to 15 seconds after the beginning of the injections, the pressure began to fall, and reached the minimum values in from 30 to 70 seconds. This level was maintained for a period varying from 30 to 90 seconds. The pressure then begins rising and levels off in 2.3 to 3.3 minutes, at a value referred to as "immediate recovery." The table shows all figures and the means and standard deviations where feasible. The mean for the minimum levels reached just after the injections is 74.0 per cent of the initial pressure. The standard deviation (S.D.) is small. The mean of the immediate recovery levels is 96.3 per cent of original. Thereafter there is a downward trend as revealed by recording the pressure 8 minutes after injection. The mean value at that time is 85.9 per cent of pre-injection blood pressure. There was no further decrease.

The response of a dog (No. 3) with only a local anesthetic, procaine hydrochloride, and of one which was dehydrated, and in peripheral vascular collapse (No. 10) associated with an obvious infection resembling distemper, are also shown. The results in three cats and two rabbits, also under ether anesthesia are included. Comparisons of the effects in these groups of animals may also be seen in Figure 4.

Discussion. All animals exhibited an immediate

reduction of blood pressure upon intravenous injection of sodium glycollate nitrate. It may be seen that only a few of the animals tested over completely recovered. All showed a secondary lowering of pressure after the maximum recovery. The one dog (No.3) which was awake when the drug was injected suffered a deeper fall, but a faster and more nearly complete recovery. Thus it can be said that the drug is quite active in the conscious dog. It was longer acting in the sick dog, No. 10.

The cats were somewhat more sensitive to the drug than were healthy dogs. The rabbits were the most sensitive of the three species studied. Their maximum recovery, 62 and 66 per cent of original pressure, was attained only after 20 and 16 minutes respectively. This greater sensitivity of rabbits to this water soluble nitrate led to the speculation that these animals might possess a poorer syster of vasoconstrictor reflexes than the other species. This might explain why their blood pressure remains quite low for a much longer time than that of the other animals.

Depressor Action of Sodium Glycollate Nitrate as Second Intravenous Injections

Results. The effects of the repeated injections are shown in Table 8 and Figure 5. The responses to the first dose are again shown for proper comparison. In the table, the original blood pressure of each animal is given in the first column. All other results are expressed as their

TABLE 8

Depressor effects of the nitrate of sodium glycollate. Secondary intravenous injections.

Dog No.	Blood Pressure			
	Before injection	Minimum reached after injection		Immediate recovery
	mm. Hg	mm. Hg	Per cent	Per cent of pre-injection level
1	115	111	96.7	99.5
2	150	142	94.7	96.0
8	124	122	98.4	98.4
5	106	100	94.5	100
Mean			96.1	98.5
S. D.			1.8	1.8

Ether anesthesia. Carotid artery blood pressure. Dose as in initial injections: 54 mg. in 0.75 cc per kg.

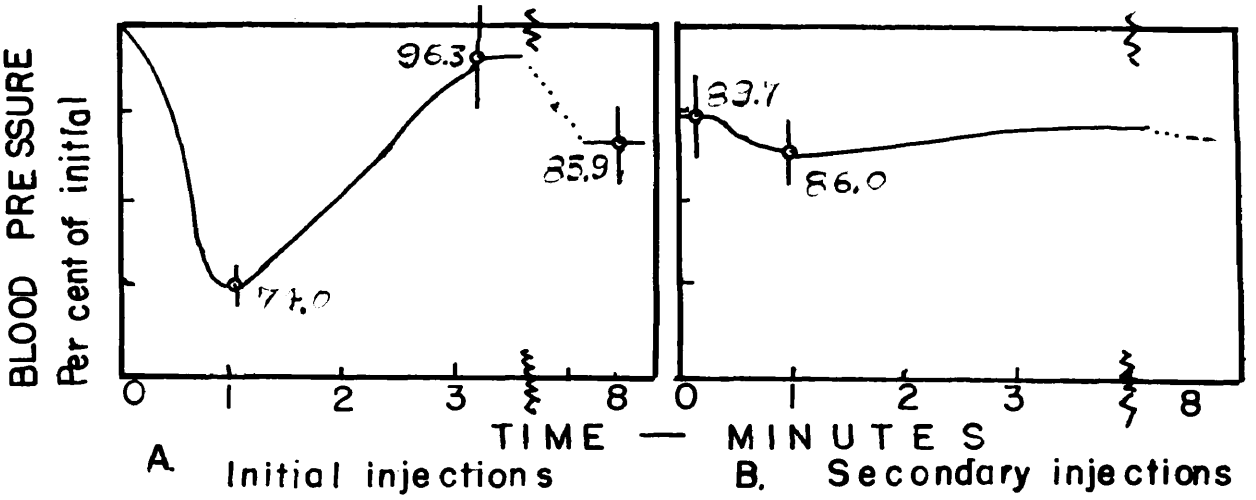

FIG. 5. COMPARISON OF EFFECTS OF INITIAL AND SECONDARY INJECTIONS OF THE NITRATE OF SODIUM GLYCOLLATE

Ether. Dose: 54 mg. per kg. Record in A represents mean initial response of dogs No. 1, 2, 8 and 117 (see Fig. 4) Record in B represents mean response of dogs 1, 2, 5 and 8 (see Table 8). The curve for the latter group was drawn on the same scale, representing secondary responses as per cent of original blood pressure. Standard deviations (vertical lines) in A are given in Table 7; those in B are 6.0 and 4.4 before and after, respectively.

per cent of that original value. In the figure, all results are given as per cent of original pressure.

The pre-injection blood pressure levels for the second doses give a mean of 86.2 per cent of the original level. It is possible that it is lower than normal because of continuing action of the first dose. The actual falls resulting from the second dose are significantly less than first responses. Third and subsequent doses rarely cause any depression of pressure whatever, and usually cause slight transient elevations.

Two cats gave results showing resistance to second and subsequent injections quite similar to that of dogs, but a third cat gave responses showing refractoriness only after the fourth dose. The percentage falls in the latter animal caused by the four doses were 36.4, 21.6, 13.4, and 8.2 per cent respectively. Two rabbits were refractory to second injections, one having recovered but slightly from the first. The transient pressor effect was frequently seen in these animals also.

It therefore appears that resistance to subsequent injections is well developed in these animals, with the exception of one cat, in which resistance was developed more slowly.

In investigating the length of time during which the remarkable resistance following the first dose might last, dogs were given a first injection while unanesthized. Later

they were given a second with the usual recording of blood pressure. The interval was varied from 2.5 to 18 hours. Table 9 shows the results of four such experiments. Resistance lasts more than 6 hours, and less than 18.

It has been repeatedly seen that alkyl esters of glycollic acid nitrate produce their characteristic definite, sharp transient depression of blood pressure as readily in the dog resistant to the sodium salt, as in the fresh animal. Furthermore, the dose of alkyl esters producing such dependable effects is far less than the threshold dose of salt given as initial injections. Concentrations of the sodium salt of the order of 0.5 molar are required in order to elicit responses in all animals, while 0.004 to 0.01 molar concentrations of the various esters produce equal falls, although of shorter duration. Thus refractoriness to the salt does not prevent response to esters. Refactoriness to esters cannot be produced in any animal that is not in shock.

Discussion. It is theoretically possible for an animal to offset some or all of the dilating tendency of a nitrate or nitrite by means of increasing vasoconstrictor tone. An experiment of Filehne's (26) long ago would support such compensatory capacity. He found that if the rabbits sympathetic nerve be cut on one side of the neck, and then stimulated by an interrupted current so as to maintain a normal degree of vascular contraction in the ear, amyl

TABLE 9

Duration of resistance to sodium glycollate nitrate following a first dose of 54 mg. per kg.

Dog No.	Interval between injections	Blood Pressure		
		Before second injection	Minimum after second injection	
	Hours	Mm. Hg	mm. Hg	Per cent
6	2.5	79	70	89
7	4	105	100	95
9	6	112	110	98
5	18	110	74	67

First doses given intravenously without anesthesia or recording of blood pressure. Second doses (equal to first) given intravenously under ether anesthesia. Carotid blood pressure recorded.

nitrite does not produce vascular dilation on that, but on the other side.

The dose of sodium glycollate nitrate given, 54 mg. per kg., is large, compared to the effective doses of other organic nitrites and nitrates. One-hundredth milligram glyceryl trinitrate per kg. will markedly lower blood pressure on intravenous injection. The concentration of the foregoing salt in the extracellular fluid of the body may be estimated, if one assumes that it is evenly distributed before it is decomposed or excreted. The volume of extracellular fluid (including blood plasma) is roughly 20 per cent of the body weight, or 200 cc. per kg. If 54 mg. per kg. of the nitrate are injected, this amount will be present in each 200 cc., or 27 mg. per 100 cc. This level is approximately the same as that of urea. If the nitrate is stable in the body, it is easily appreciated that hours would be required to eliminate the major portion of a single dose via the kidneys. If the nitrate be decomposed as quickly as the blood pressure recovers, it is difficult to see shy the animal is not reactive to a second dose. The compound is very stable to alkali hydrolysis. It does not explode on heating. We may expect it to be relatively stable in blood for the following reason. Oberst and Snyder (56) found that the blood was able to decompose only a slight amount of glyceryl trinitrate, whereas a liver homogenate was able to decompose much more. It is reasonable to expect

greater chemical activity toward a nitrate in the body cells than outside them. A moderate degree of decomposition of sodium glycollate nitrate could progress in the blood and interstitial fluid and yet not rapidly reduce the concentration of the agent.

The low oil over water solubility coefficient of this nitrate does not favor its permeation of the body cells where rapid decomposition may be expected. It can be expected largely to stay in the extracellular fluid. Unfortunately no dependable tests for this compound are available whereby blood and urine concentration of the agent could be measured. This would reveal the validity of the hypothesis that a substantial blood and extracellular fluid level of it is maintained for a protracted period of time.

Depressor Action of Sodium Glycollate Nitrate Upon Continuous Slow Infusion

The resistance or refractoriness of dogs to the second and subsequent doses of the sodium salt suggested the trial of a continuous intravenous infusion of the agent.

Dog No. 14 under ether anesthesia received a 0.5 molar solution for 13 minutes, at an approximate rate of 0.4 cc. per kg. per minute. The blood pressure fell from 112 mm. Hg. to a minimum of 73 per cent of that level in two minutes. It then began rising, reaching 93 per cent of normal within 5 minutes. At the end of the injection it had receded to

84 per cent of original. The pattern followed is quite similar to the average response to single injections already described. Such an injection now, of the usual dose, 0.75 cc per kg. had a slight pressor effect. One fiftieth of that dose (0.01M.) of the heptyl ester then had its usual effect, a drop in blood pressure to 72 per cent of original with rapid recovery. The tolerance again did not extend to the ester.

Depressor Action of Sodium Glycollate Nitrate After Injections of Autonomic Nervous System Blocking Agents

It seemed appropriate to paralyze, if possible, the sympathetic nervous system before administering the glycollate nitrate salt. It has been mentioned that a sick dog (No. 10) and two rabbits gave a much more prolonged depressor effect than did healtny dogs. The marked difference in the latter animals could possibly be a result of inadequate vasoconstrictor reflexes in them. The recovery of healthy dogs and cats may be caused by such homeostatic mechanisms. The nitrate, even though still present in blood and intercellular fluid, may be too weak to act on the smooth muscle fibres when they are being stimulated neurogenically.

Table 10 shows the results in two dogs of nicotine injections, and of subsequent intravenous administration of sodium glycollate nitrate. In dog No. 11 the usual dose (54 mg. per kg.) of nitrate as previously employed was

TABLE 10

Effect of nicotine on the depressor activity of sodium glycollate nitrate.

Dog No.	Injection No.	Dose of nitrate mg./kg.	Blood Pressure: Prior to injection Mg. Hg	After injection 1 minute Mg.Hg	%	After injection 8 minutes Per cent
11	1	54	104	68	65	71
12	1	5.4	92	77	84	70
	2	5.4	72	68	94	
	3	5.4	72	68	94	
	4	108	72	72	100	

Intravenous injections. Ether anesthesia. Carotid artery pressure recorded. Doses of nicotine. Dog No. 11, 1.67 mg. per ke.; Dog No. 12: 1 mg. per kg. Depressor response to stimulation of vagus was nearly abolished. Blood Pressure before nicotine: Dog No. 11: 148 mm.; Dog No. 12: 106 mm.

given. A deep fall in pressure was produced which was long lasting and thus is in contrast to the short falls shown in Table 7. It more resembles the effect produced in the sick dog (No. 10) and in the rabbits. In dog No. 12, one tenth of the former dose of the nitrate was given, and again, a prolonged depressor effect was obtained, although the immediate fall was less than usual. This small dose, 5.4 mg. per kg., does not produce more than a faint effect in animals not premedicated with nicotine. It was repeated twice and slight further depression only was produced. This suggests that in this animal, the first small dose was sufficient to produce a near maximal effect. Now a larger dose than ever, 108 mg. per kg., was incapable of lowering the pressure further. Figure 6 shows graphically the results of the initial injections in 2 animals. In one experiment, Dibenamine, 20 mg. per kg. intravenous, was used as the sympathetic blocking agent. A long continued depression of blood pressure was produced when the reduced dose of the nitrate (5.4 mg. per kg.,) was injected.

Depressor Action of Sodium Glycollate Nitrate Upon Injection Into The Small Intestine

The absorption of sodium glycollate nitrate was studied in several animals by observing the arterial pressure when the drug was injected into the lumen of the gut through a hypodermic needle. The abdomen was opened in the mid line and a loop of small intestine picked up with the fingers.

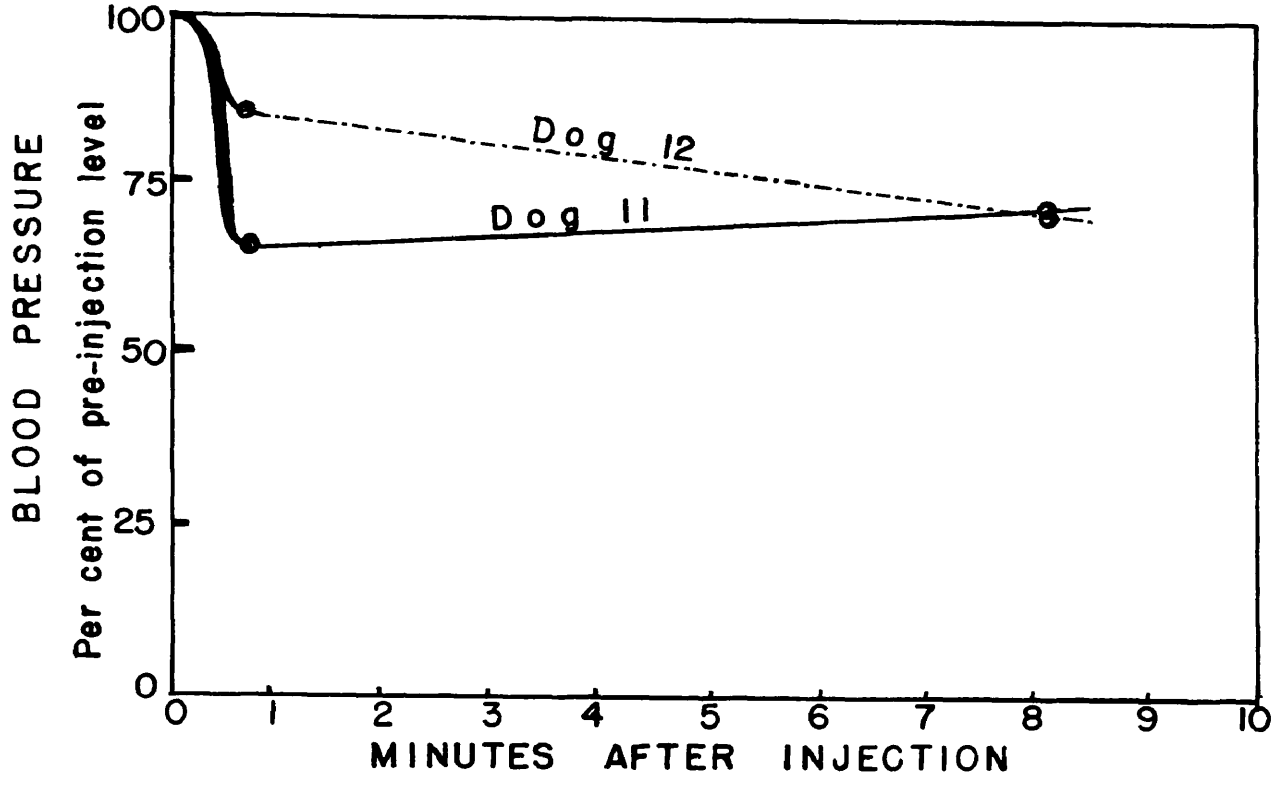

FIG. 6. GRAPHIC REPRESENTATION OF RESULTS OF TEN INTRAVENOUS INJECTIONS GIVEN IN CALL 10 USING NICOTINE PRETREATMENT

Saline injection was made as controls for this traumatic procedure. In no case was a fall produced which vitiated the significance of responses to drug injection. Either slight transient falls were seen as controls, or none were observed at all.

The intestine was chosen for the reason that more rapid action should ensue than would be the case if the stomach were inoculated. Table 11 shows the responses obtained in three dogs and one rabbit. The onset of action (depressor effect) was definite enough in three cases to show that the drug is active by this route. The variation in susceptibility is evident from the data.

The resistance or even refractoriness seen after intravenous injection of this nitrate was again produced by these intestinal injections. When intravenous injections were given after the latter, slight rises in pressure were produced, and no further depression could be achieved.

One dog (No. 20) was given Dibenamine. A small dose of the sodium salt of the nitrate of glycollic acid was injected into the intestine after a saline control had caused no change in blood pressure. The dose employed, 5.4 mg. per kg., gave no appreciable fall when given intravenously to animals which had not received Dibenamine.

In this experiment the pressure immediately began a slow decline, dropping from 120 to 84 mm. Hg in 7 minutes. It remained at this level for another 8 minutes, at which

TABLE 11

Activity of sodium glycollate nitrate upon injection into the small intestine

Animal	Dose	Blood Pressure		
		Initial	10 minutes after injection	
	mg./kg.	mm. Hg	mm. Hg	Per cent
Dog 15	96	122	106	87
Dog 16	144	130	125	96
Dog 114*	108	116	88	76
Rabbit 3	54	80	58	73

Ether anesthesia. Carotid artery pressure recorded.
*Dog 114 had previously received other depressor drugs but blood pressure was constant at 116 mm. Hg. Effect of nitrate rapid in this animal.

time an intravenous injection identical to the first was made. Table 12 shows the results of these and subsequent intravenous injections. A step-like depression can be accomplished by employing Dibenamine and small repeated doses of the nitrate. No resistance or refractoriness to the drug is seen in an experiment until low levels of pressure (wide dilatation of blood vessels) are reached. It can even be seen that there is apparently a dose - response relationship with an accumulative effect.

Discussion. Evidence that sodium glycollate nitrate acts by the intestinal route has been presented. This drug is weakly depressor unless the adrenergic sympathetic outflow is weakened or paralyzed. Prolonged falls are then obtained.

Other Effects of Sodium Glycollate Nitrate

The pulse rate usually increases during the depressor response to this nitrate, just as it does after injections of the alkyl esters of glycollate nitrate and of the sodium derivatives of the latter. An electrocardiogram taken before and during the depressor action showed no abnormality.

Respiration is usually not significantly changed. A slight decrease of excursion occasionally accompanies the hypotension.

In two experiments the spontaneous rhythmic contractions of a guinea pigs intestinal segment in vitro were immediately abolished by addition of 7 mg. to a 50 cc. bath. This is of

TABLE 12

Effect of Dibenamine on depressor activity of sodium glycollate nitrate given by intestinal and intravenous routes

Time Minutes	Agent	Route	Dose	Blood Pressure mm. Hg
0				120
0	Dibenamine	i.v.	20 mg./kg.	120
5	Physiol. Saline	gut	0.75 cc./kg.	120
10	Nitrate	gut	5.4 mg./kg.	120
11				declining
17				84
25	Nitrate	i.v.	5.4 mg./kg.	83
25.5				76
30	Nitrate	i.v.	5.4 mg./kg.	76
30.5				72
32	Nitrate	i.v.	5.4 mg./kg.	72
32.5				68
36	Nitrate	i.v.	10.8 mg./kg.	68
36.5				65
75				63

Dog No. 20

course typical of nitrite and nitrate action.

TOXICOLOGY

Acute Toxicity

Dogs which received 54 mg. per kg. of the nitric ester of sodium glycollate intravenously and unanesthetized, behaved in a normal manner and exhibited no observable ill effects. Membranes of mouth and eyes had their normal color. This was the dose used in studying the depressor response to the drug.

Ten pairs of rats weighing 140 to 200 grams were given, intraperitoneally, graded doses which were multiples of 70 mg. per kg. The highest dose was 840 mg. per kg. All animals survived the 20 hour observation period. None exhibited abnormal behavior or appearance save one which had a soft stool within 20 minutes after injection of 840 mg. per kg. Cyanosis was not seen in these animals.

The intraperitoneal LD_{50} for rats was not found below 3 grams per kg., hence was not determined.

Chronic Toxicity

Six rats were injected intraperitoneally once a day, 29 out of 39 days. One pair received a dose of 80 mg. per kg., another 160, and a third, 320 mg. per kg. An additional pair served as controls, receiving physiological saline solution in the same volume as used for the largest dose of

drug. All animals increased in weight as may be seen in Table 13.

The blood cells were studied in the rats receiving the two higher doses employed, and in one control animal, after the conclusion of the foregoing experiment. The abnormal findings are listed in Table 14. The blood of all rats, including the control, revealed low counts of the red cells and low content of hemoglobin. The leukocyte counts varied greatly, as do those in normal rats, but nearly within normal limits. Differential counts of the leukocytes were also normal. Inflammatory reactions and hematoma of the abdomenal wall may have been partly responsible for abnormal blood pictures.

A larger dose, one gram per kg. was given daily to 5 rats by intraperitoneal injection. Their weights ranged from 125 to 165 grams. After ten injections in eleven days the weights were found to have decreased from 0 to 9.4 per cent of initial values. One rat died on the fifth day and two on the twelfth. The two remaining were killed on the thirteenth day. The following organs were sectioned, stained, and examined: heart, lung, liver, kidney, spleen, intestine, bone marrow, and testicle. In one of these animals, all of the foregoing specimens were reported to be normal. In the second rat, infectious lesions of the intestine, spleen and liver were seen. This may have been incident to the injections (contaminating organisms).

TABLE 13

Effect of sodium glycollate nitrate on weight
29 intraperitoneal injections in 38 days in rats

Rat No.	Dose mg./kg.	Initial kg.	Terminal kg.	Per cent each	Increase average
1	80	0.260	0.280	7.7	
2	80	0.227	0.260	14.8	11.1
3	160	0.256	0.270	18.7	
4	160	0.220	0.300	36.4	27.6
5	320	0.245	0.290	18.3	
6	320	0.244	0.270	10.7	14.5
		Overall average		17.8	
7	Control	0.240	0.290	20.8	
8	Control	0.240	0.300	25.0	22.9

Volume injected: 0.68 to 2.7 cc. per 100 gm.

TABLE 14

Effect of sodium glycollate nitrate on blood elements

Rat No.	Erythrocytes millions/cc.	Hemoglobin grams per 100 cc.	Leukocytes per cc.
3	8.08	11.6	8,300
4	6.95	11.7	8,500
5	4.58	8.0	20,800
6	6.40	10.5	7,700
7	7.70	7.7	19,900
Normal *	9	15.6	8,800 to 15,000

Rats are those described in Table13. *Literature

CHAPTER VII

DEPRESSOR ACTIVITY OF THE NITRIC ESTERS OF MISCELLANEOUS HYDROXY ACIDS

Malic Acid

The disodium salt of the nitric ester of malic acid was prepared. It is quite soluble in water. Solutions of 1 M. and 2.7 M. strength were injected intravenously 9 times in 4 dogs.

Doses of 6 mg. per kg. produce a slight rise in pressure with rapid recovery. Larger doses, between 12 and 167 mg. per kg. produced immediate falls, none lower than 86.8 per cent of pre-injection blood pressure. In all cases, however, secondary rises above normal occurred. These varied from 7 to 25 per cent greater than pre-injection levels. In 60 seconds recovery at the latter levels was seen.

Tartaric Acid

The monosodium salt of the dinitrate of tartaric acid, with pH of 5, was injected intravenously into two dogs. Only pressor responses were obtained in one, and preliminary depressor effects with secondary rises were seen in the second. Doses from 65 to 194 mg. per kg. were used.

The disodium salt, with a pH of 8.5, was injected into one animal. Elevations of blood pressure of less than 5

per cent were at first produced, followed by a depression to 88 per cent of initial pressure within two minutes. A slow partial recovery over a ten minute period followed. These effects were caused by a dose of 25 mg. per kg. A dose 50 per cent greater nearly duplicated the results in the same animal.

alpha-Hydroxy Isobutyric Acid

The nitrate of the ethyl ester of this acid was prepared in 0.01 M. solution in 10 per cent alcohol. 0.1 cc. per kg. gave a deep transient fall in blood pressure when injected intravenously into a dog. 0.75 cc. per kg. was survived once, with considerable depression of respiration, but was fatal when repeated. Cardiac and respiratory arrest was immediate.

Paroxysmal tachycardia was produced in a rabbit after an intestinal injection of 1.5 cc. per kg. of the same solution. The toxic effects thus observed made further observations appear to be unwarranted.

Mucic Acid

The tetranitrate of mucic acid was prepared as the disodium salt. A 0.17 M. solution, at pH 8.5, was injected three times into two dogs. 0.1 cc. per kg. gave no noticeable effects. 0.75 and 1.0 cc. per kg. gave transient pressure effects of moderate degree.

Saccharic Acid

The dinitrate of the disodium salt of saccharic acid was prepared as a 0.1 M. solution. One cubic centimeter, containing 34.4 mg., was injected intravenously for each kilogram. Four injections were made into two dogs. The blood pressure fell to values ranging between 77 and 86 per cent of the pre-injection levels. A cumulative effect was observed. The compound is definitely depressor, but the dose required is large. It seems to be more potent than sodium glycollate nitrate. No tolerance to second injections was seen, and the hypotension caused lasted for more than 30 minutes.

Lactic Acid

Ethyl lactate nitrate in saturated aqueous solution was injected into a dog intravenously, 1.0 cc. per kg. An immediate fall in blood pressure to 84 per cent of the former level was observed. A rapid recovery ensued. The response was very similar to that following alkyl glycollate nitrate injections. Krantz, et al. (47), observed the potent depressor activity of this lactate. They found the sodium salt of lactic acid to be very weak, and commented on the great difference in the two compounds.

Glyceryl l-glycollate

The trinitrate of glyceryl l-glycollate was synthesized and injected twelve times into four animals. It proved to

be roughly equivalent to glyceryl trinitrate in potency but weaker than mannitol hexanitrate on a molar basis. Increased duration of activity was not attained by the new nitrate. It was sparingly soluble, and, like glyceryl trinitrate, it was injected in alcoholic solution. The pure compound, in oil, was quite active when applied to the oral mucous membranes of dogs.

1, 3-Diacetyl glycerol was nitrated at the number 2 position and a 0.02 M. solution in 20 per cent alcohol was injected intravenously. A 20 per cent fall in pressure occurred immediately. Recovery required 90 seconds.

Discussion

No sparingly soluble organic nitrates were found which exhibit any superiority over the polynitrates such as mannitol hexanitrate. The four esters investigated possess depressor activity in small doses. The responses are temporary, and another dose produced approximately the same results.

Some of the salts of nitrated acids (saccharate and tartrate) possess depressor activity of a prolonged type, but only when considerably larger doses are given. They are quite water soluble.

CHAPTER VIII

INVESTIGATIONS OF SOME POSSIBLE MECHANISMS OF ACTION OF NITRATE AND NITRITE

ADENOSINE TRIPHOSPHATE ACTIVITY

The immediate source of energy for skeletal muscle contraction is described as being the high energy phosphate bonds in adenosine triphosphate (ATP). The hydrolysis of this compound is catalyzed by certain enzymes having the collective name adenosinetriphosphatase (ATPase). Myosin, the contractile protein of skeletal muscle, has high ATPase activity. This is a strategic arrangement. There are enzymes in various non-muscular tissue which hydrolyze ATP (21), and there has been described a non-myosin ATPase in skeletal muscle (43). Certain ions have marked effects upon the activity of these enzymes. Magnesium and copper strongly inhibit the activity of myosin on ATP. Chloromercuribenzoate has been found to inhibit the hydrolysis. Other inhibiting substances found in biological materials have been described.

No mention of studies of the effect of a nitrate or nitrite upon ATPase activity was found. Sodium nitrite was selected as the appropriate representative of the group for such a study. Because more has been learned and described concerning ATPase activity of skeletal muscle than

of smooth muscle, most of the experiments herein reported were made with skeletal muscle preparations.

Method

The manner of preparing a homogenate of skeletal muscle was one described by Dubois and Potter (21). A rat was killed by a blow on the head and the thigh and gluteal muscles on one side were quickly removed and macerated in a cold tissue mincer. The pulp was quickly weighed and then ground with cold, washed sand in a cold mortar. A paste-like consistency was attained with sand for the first five minutes of grinding, to insure better disintegration of muscle fibres. Cold distilled water was gradually added until a one per cent suspension of muscle was obtained.

The substrate for the reaction was sodium ATP, prepared from the commercially available dibarium ATP. The conversion was accomplished by passing a solution of the barium salt through a column of Amberlite IR-100 resin which had been "activated" by treatment with sodium carbonate. The ion exchange procedure was repeated once. The eluate, sodium ATP, barium free, was brought to a pH of 7.4 with hydrochloric acid and to such volume as to result in a 0.013 M. solution.

The hydrolysis of ATP by the muscle homogenate was carried out with small quantities carefully measured, using appropriate pipettes. Glass tubes 10 x 50 mm. were used for incubation of the materials. The reagents listed below are

as described by the foregoing authors, but the amount of water added has been increased in order to facilitate subsequent pipetting. Increased accuracy was attained thereby. Into some tubes were placed the following solutions:

0.15 cc. 0.05M. diethyl barbiturate, pH 7.4
0.05 cc. 0.04M. calcium chloride
0.15 cc. 0.013M. ATP, pH 7.4 (intentional excess)
0.10 cc. 0.01 or 1.0 per cent $NaNO_2$ solution
0.10 cc. 1.0 per cent muscle homogenate
0.35 cc. distilled water

Two or three tubes were thus prepared. Two or three more were prepared omitting the sodium nitrite, replacing it with an equal volume of water. In two tubes only the muscle suspension (0.10 cc) was placed, with 0.8 cc. water to bring to a volume equal to that of the others. This was the procedure used in every experiment. After fifteen minutes incubation at 37.5 C., to each tube was added 0.1 cc. 50 per cent trichloracetic acid to halt enzymatic activity. One cubic centimeter of water was then added, bringing the volume in each tube to 2.0 cc. Suspended matter was avoided by centrifuging, and inorganic phosphate determinations were made using 1.0 cc. portions of the supernatant fluid. The figures obtained were then doubled, in order to represent the original reaction mixtures. The original method of Dubois and Potter called for a final volume of 0.65 cc., from which 0.30 cc. was removed for analysis. The mechanical difficulties of pipetting the latter without disturbing the sediment was objectionable. Errors in pipetting the aliquot have less effect on variation of results if the

greater dilution is made.

Frequently during the weeks during which this study was made, analysis of the phosphate contained in the buffer, the calcium chloride solution, and the ATP solution was determined. The figures varied only slightly during that time from 3.9 to 4.7 gamma inorganic phosphate, contained in all three solutions combined, in the amounts placed in the incubation tubes.

All necessary control data have now been described. By subtracting the phosphate of the ingredients (buffer calcium chloride, and ATP, analyzed together, and muscle homogenate analyzed alone) from that of the mixture of all reagents except nitrite, the normal activity of the muscle enzymes can be ascertained. The same may be done when nitrite is present, and the effect of the latter readily seen. The first four experiments were run in pairs, and the last three in triplicate.

The method of determining inorganic orthophosphate was modified from Fiske and Subbarow (28). It is a colorimetric procedure in which the unknowns are compared to standards in an photoelectiric colorimeter. A Klett-Summerson instrument was used in this work. A blank was run in each case, using only the reagents for developing the color. The readings on the scale were substracted from those of the standards and of the unknown solutions before the latter two groups were compared. Phosphate present in the color reagents and

distilled water were thus controlled. Readings were taken at least twice, and if color had deepeded, owing to further hydrolysis of ATP in the acidic analytical reagents, extrapolation to zero time was made.

In one experiment (Number 5) a solution [1] of rabbit skeletal muscle proteins, with other cellular constituents, derived by extracting without grinding was used. Amberson (1) devised a "mild extraction" procedure using a pyrophosphate splits myosin away from its combination with actin, whereupon it diffuses into the solution through the intact sarcolemma. The readings produced in analyzing for phosphate were higher than necessary, because the solution was not dialyzed after extraction. This did not affect final results.

In another experiment (Number 6) the same extraction method was applied to dog urinary bladders. The resulting solution was dialyzed against distilled water. The precipitated proteins were then partly redissolved by adding crystalline potassium chloride calculated to result in a 0.12 molar solution of that salt. Myosin is most soluble in dilute KCl solutions. The myosin content of the solutions thus made was not known quantitetively. ATPase activity was demonstrated however.

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[1] Kindly supplied by Dr. W.R. Amberson, Professor of Physiology, School of Medicine, University of Maryland.

The amount of sodium nitrite used in the first four experiments was 1 mg. (1.0 per cent solution). This amount, and an equal amount of wet muscle, were diluted nine times during the incubation. In the last three experiments the nitrite concentration was only one hundredth of that in the preceding ones. The latter would be closer to a theoretical concentration of nitrite attainable in vivo with a depressor dose. It seemed advisable to employ two drug levels in order to ascertain any differences in effects which might exist.

Results

All actual readings on the colorimeter, made at ten minutes after color reagents were added, are given in Table 15. The variability in duplicate and triplicate determinations may thus be seen. The table also presents the final amounts of phosphate, as phosphorus, liberated with and without sodium nitrite. These figures have been corrected for free phosphate in all reagents and for that in the muscle, or produced by it, during incubation, from its inherently bound phosphate.

Discussion

In three experiments, there seemed to be greater enzyme activity in the presence of sodium nitrite than in the normal controls. This may be insignificant. It was shown not to be caused by phosphate contaminating the nitrite by several analyses on that solution alone. When the amount of nitrite

TABLE 15

Effect of sodium nitrite on adenosinetriphosphatase activity of muscle tissue

Experiment No.	Source of ATPase	ATP hydrolysis in 15 minutes at 37.5°C			
		Colorimeter readings		Phosphorus liberated	
		Normal	With $NaNO_2$	Normal	With $NaNO_2$
				gamma	gamma
1			89.5 91.0		15.6
2	1.0 mg. wet skeletal	57.0 61.0	71.0 (lost)	11.4	14.5
3	muscle (rat) homo-	66.5 67.0	76.0 75.5	12.5	15.0
4	genized in 0.1 cc.	97.5 89.0	107.0 101.0	17.6	20.0
5	water	97.5 100.0 98.5	87.5 92.0 102.5	19.4	18.1
6	0.1 cc. skeletal muscle extract	166.0 160.0 175.0	166.0 170.0 178.0	6.7	6.5
7	0.1 cc. smooth muscle extract	45.0 44.0 44.0	44.5 44.5 44.0	6.4	6.4

1.0 mg. $NaNO_2$ used in experiments 1 - 4; 0.01 mg. used in remaining ones. Extracts in experiments 6 and 7 made by suspending muscle tissue without grinding in pyrophosphate solution at 0°C for several days. See text.

used was decreased, there were less differences between the treated and non-treated samples. The greater agreement may have resulted from the employment of three samples for each group. More accurate mean figures are to be expected when more determinations are made. It was impractical to add more samples to each experiment. The other controls and standards, added to the six samples, brought the total number of analyses to twelve or fourteen. The lability of ATP in acid solution necessitated accurate timing when making readings. More analyses cannot be made with the rigid schedule which must be followed.

It is concluded that sodium nitrite has no effect upon the ATPase activity of skeletal or of smooth muscle enzymes in vitro. A single experiment using Syntropan (tropic acid ester of 3-diethylamino - 2, 2 - dimethyl - 1 - propanol),4 γ per tube revealed no effect upon ATP hydrolysis by the smooth muscle extract, This is ten times the concentration which will relax a smooth muscle strip in a bath.

OXYGEN CONSUMPTION OF KIDNEY IN VITRO

No studies on the effect of a nitrite or of a nitrate upon the oxygen uptake of surviving tissue cells were found. It was considered desirable to determine whether a change in the respiration occurs in the presence of one of those agents. Sodium nitrite offered the best characteristics, being inorganic, neutral, simple in composition, and stable in solution.

Method

Albino rats of both sexes weighing 130 to 200 grams were used. They were fed a standard diet to which they had free access. They were killed by a blow on the head. The kidneys were bisected longitudinally through the hilum. The capsule, pelvis structures, and pyramids were removed, leaving the cortex and a subjacent thin shell of medulla. Slices were then cut 0.2 to 0.3 mm. in thickness and kept in cool Ringer-Locke solution. The poles of the organs were not used, in order to maintain a more uniform proportion of cortex and medulla in the slices.

Into each respirometer flask containing 3.0 cc. Ringer-Locke solution were placed 4 or 5 slices of tissue, selected for uniform thickness. In the center wells were placed 0.2 cc. 20 per cent potassium hydroxide solution and a measured size strip of special filter paper. The latter served to increase the surface area of the alkali and thus to promote adequate absorption of carbon dioxide. The gas phase was oxygen, established by a 5 minute flushing with the gas. A period of 15 minutes shaking with stop-cocks open in the water bath, maintained at 38.0 $\pm$ 0.1°C. Was allowed for temperature equilibration. The interval between the time of death of the animal and the beginning of measurement of respiration varied between 45 and 60 minutes. The shaking apparatus produced 110 complete oscillations per minute through a distance of 4.5 cm.

This constitutes the direct method of Warburg as modified and thoroughly explained by Dixon (18). Other details of the method, including materials and calculation of flask constants, were followed as presented by that author.

In each experiment, three flasks served as controls, measuring oxygen consumption under the conditions described, without the addition of a drug. Three flasks served to measure oxygen uptake in the presence of the nitrite. One flask, without tissue, served as a control of the effects of temperature variations on the volume of gas in the flasks. Carbon dioxide was absorbed and thus did not affect the reading of oxygen uptake. The latter may be referred to with the customary symbol, Q_{O_2}. It will be expressed as cubic millimeters of the gas at normal temperature and pressure, which were consumed by the tissue in terms of mg. (dry weight), per hour. To convert such values to a basis of wet tissue weight a multiplication factor of 4.88 ± 0.12 has been offered by Crimson and Field.

The three determinations for Q_{O_2} in the presence of nitrite were averaged, and the mean was expressed as per cent of the mean of the three determinations of the normal Q_{O_2} for that animal. Thus 100 per cent Q_{O_2} would mean that there was no significant difference in oxygen consumption between the controls and the drug-treated tissue. 50 per cent Q_{O_2} represents a reduction in oxygen uptake by that

cent Q_{O_2} represents a reduction in oxygen uptake by that

Normal Q_{O_2} in these experiments varied between 8.1 and 15.3.

Results

The results of the experiments using sodium nitrite are summarized in Table 16. The data are divided in order to show in the upper portion those obtained by the author with R. Burgison, and in the lower portion those obtained by J. Knapp.

From the table, it can be seen that smaller concentrations of sodium nitrite do not measurably decrease oxygen uptake, while concentrations above 0.05 per cent definitely depress Q_{O_2}. There was very little variation in the figures for the Q_{O_2} in the flasks containing 0.05 per cent or more nitrite, as seen in Table 17.

In a single experiment using slices of small intestine without stripping its mucosa, Q_{O_2} was depressed to 58 per cent of control values in the presence of 0.10 per cent sodium nitrite. Sodium nitrate, isosorbide dinitrate, and isobutyl glycollate nitrate did not significantly suppress oxygen uptake of intestine. All were present in 0.10 per cent concentration. The latter glycollate had no inhibitory effect on kidney slices in the same concentration.

Discussion

The concentration of sodium nitrite which inhibits oxygen consumption of kidney cells <u>in vitro</u> is so great

TABLE 16

The effect of sodium nitrite on the oxygen consumption of kidney slices

	Concentration of sodium nitrite					
	0.01%	0.018%	0.022%	0.025%	0.05%	0.10%
Q_{O_2} as per cent of controls	100%	100%	100% 79%	90% 67%	55% *	63%* 42%*
Averages	100%	100%	90%	89%	55%	53%
Q_{O_2} **				100% 100%	57% 42%	59.1%

*Individual Q_{O_2} determinations making up these results are exhibited in Table 17. ** Additional determinations (see text).

TABLE 17

Effect of sodium nitrite on Q_{O_2} of kidney slices: individual determinations

Concentration of $NaNO_2$	Q_{O_2} - Cu. Mm. per Mg. dry weight				Q_{O_2} with $NaNO_2$, as per cent of control
	Controls		With $NaNO_2$		
	Each flask	Average	Each flask	Average	
0.05%	10.0 10.3 (lost)	10.15	5.3 5.2 6.2	5.57	55
0.10%	11.3 15.0 11.9	12.73	7.5 8.1 8.6	8.07	63
0.10%	11.1 12.7 12.1	11.97	4.4 5.7 5.0	5.03	42

that it cannot be said that these experiments serve to demonstrate any effect on Q_{O_2} which may occur <u>in vivo</u> with any reasonable depressor dose. The large doses given by Rath (op. cit.), and repeated in work previously described herein, would give a theoretical extracellular fluid concentration of about 3 mg. per cent. No inhibition of Q_{O_2} was observed in solutions containing 10 mg. per cent.

The effect of sodium nitrite on resting skeletal or smooth muscle could not be interpreted as reflecting the effect on contracting muscle. The oxygen consumption of smooth muscle in a state contraction could not be measured in the presence of nitrite because the muscle quickly relaxes in contact with that drug. The oxygen requirement of the muscle would of course drop to a fraction of its former value, because it is no longer working.

It is concluded therefore, that in therapeutic doses, nitrites and nitrates probably do not cause depression of oxygen uptake by any tissue except contractile tissue which is relaxed or weakened by the drug. Decrease in oxygen consumption in that case would of course be incident to relaxation. It may yet be the cause of the relaxation. Perhaps metmyoglobin is formed and thus rob the contractile machinery of one source of energy. Perhaps aerobic or glycogen metabolism is interfered with at some point. A long series of investigations would be required in exploring all possibilities.

CYTOCHROME OXIDASE ACTIVITY

It would seem reasonable to suspect that the nitrates and nitrites would be capable of oxidizing the iron of cytochrome oxidase to the ferric state, as they do that of hemoglobin. It would then be possible to detect decreased function of that enzyme. In order to determine any such inhibition, a saturated aqeous solution of n-propyl glycollate nitrate was prepared (ca. 0.18 per cent) and neutralized to a pH of approximately 8 with sodium bicarbonate.

A fresh rat brain brei was prepared using 4 cc. water to one gram of well ground brain. Two cc. of this suspension were treated with one cc. of the nitrate solution, and another such quantity of brei was used as control. One half cc. of "NADI" reagent (1) was added to each, and the tubes placed in a water bath at 37.5 $\pm$ 0.2°C. In all tubes, the color began developing in two minutes and was fully and equally developed in five minutes. There was thus no inhibition of the cytochrome oxidase in the brain suspension by the nitrate. This experiment was repeated once with the same results.

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(1) Contains dimethylparaphenylene diamine HCl, alpha-naphthol and sodium carbonate.

CYTOCHROME REDUCTASE ACTIVITY

One cc. of a 1:4 brain brei, 1 cc. of a buffered 1:5000 methylene blue solution, and 0.1 cc. of either 1.0 or 0.01 per cent sodium nitrite solution were placed in Thunberg tubes. They were evacuated of nearly all air, and placed in a bath at 37.5 $\pm$ 0.2°C. The time for decolorization was the same for the nitrite containing suspensions as for their respective controls. The time was 21 minutes with 1 per cent nitrite (control, 22) and 17 minutes for 0.01 per cent nitrite and its control.

Inorganic nitrite does not appear to inhibit cytochrome reductase even in high concentration. The higher concentration of nitrite in the foregoing tests was 50 mg. per 100 cc. of reaction mixture being incubated.

CHAPTER IX

DISCUSSION AND CONCLUSIONS

Discussion

It has been desirable to place discussion after the experimental data throughout this work. In this section, some of the concepts derived from these experiments and from the leterature will be summarized and better integrated.

When an organic nitrate which is sparingly soluble in water is injected intravenously, the threshold depressor dose is very small. It probably acts in the vascular bed of the lungs. Only a fraction of the dose injected may reach the greater circulation. Probably none reaches the venous system, because of chemical changes in its structure produced by the tissue cells. It probably reaches the cells, mainly those of pulmonary tissue, without being altered to an appreciable extent by the blood. It reaches all types of cells as nitrate, and in their plasmatic membranes or cytoplasm it undergoes chemical change. It cannot be said whether the nitrate molecule relaxes smooth muscle while it is in its original form, or whether it is a further reduction product which is responsible. Several reduction products are known, the lowest being ammonia. It is undoubtedly some reaction between one of the compounds possible in the chain of reduction, and some constituent of the cell which causes

the relaxation of smooth muscle and the weakened contraction of striated muscle. Work of the author and others have shown that the nitrates are quickly changed chemically by the body as a whole and by isolated tissue and cells. This is in contrast to the type of cellular depressants represented by volatile anesthetics which are believed not to be altered during their sojourn in the body.

Just what the reaction may be which causes depression of muscel tissue is not known. Some possibilities have been rendered unlikely by experiments reported in the preceding chapter. There are many more cellular reactions, predominately enzymatic, which could be studied under the influence of nitrite or nitrate. The search for one, on which these agents might have a depressant effect in very low quantities, would probably be a long one. Confusing results might be obtained as the interrelations of the catalytic reactions are very complex. The present therapeutic status of the nitrates may not warrant such a search.

In a water soluble organic nitrate, such as sodium glycollate nitrate, the threshold intravenous depressor dose is many times greater than that of the sparingly soluble compounds. Whether this is because its oil over water coefficient is so low that it fails to enter the lipoid membrane of the muscle cell in adequate quantity, or because of increased resistance to chemical change by the cellular systems, cannot be stated. It is probably due to both of

these factors.

With the increase in threshold dose, a decrease in toxicity occurs in water soluble nitrates. Large doses can be given which probably circulate for hours. Elimination may be by way of the kidneys and by degradation in the tissues. The latter must be slow, or of a different type than that which occurs in the case of insoluble nitrates. Otherwise we should expect more potency from the soluble compounds.

The resistance of the vascular system to repeated doses of sodium glycollate nitrate seems to be caused by an adjustment by that system to the presence of a high extracellular fluid level of the drug. It may be tolerance in the usual sense on a chemical basis, or it may be an adjustment on the basis of increased neurogenic vasoconstrictor tone. If the latter is rendered impossible by drugs which block constrictor impulses, greatly prolonged falls are obtained and with smaller doses of the glycollate.

All glycollate nitrates were active as depressor agents when injected into the gut. Sparingly soluble nitrates, if active by the intravenous route, may be placed in the gut in larger amounts whereupon continuous absorption and depressor action ensues for long periods. Mannitol hexanitrate is the most potent vasodilator of any nitrate used in this work. Erythrityl tetranitrate and glyceryl trinitrate were less potent, yet far more active than the glycollate nitrates.

These polynitrates are very sparingly soluble, particularly the six carbon compound. A depot of the latter in the gut is probably not exhausted for the 4 to 6 hours during which blood pressure is reduced by a therapeutic dose (30 mg.). The fraction of the dose finding its way into the blood stream each second reaches the cells, and that which reaches the muscle cells of the vascular tree causes relaxation. The blood concentration probably is maintained at a plateau which is extremely low, because of continuous destruction of the nitrate by the cells.

The rapid disappearance of relatively great doses of sodium nitrite after intravenous administration makes it appear futile to search for blood nitrite after administration of minute doses of the very potent polynitrates. The blood level of nitrite after administration of nitrate has occupied many workers for many decades. Marshall believed, in 1897, in the probability that any reduction which occurs to nitrates is in the cells themselves.

There is apparently no need for a more potent insoluble nitrate than mannitol hexanitrate. The possibilities of water soluble organic nitrates in therapeutics are not yet clear. They would appear to merit further research. These studies indicate low toxicity and prolonged action of a milder degree than that of insoluble nitrates. The sodium derivatives of alkyl glycollate nitrates are excluded from the promising water soluble compounds because of their methemoglobin formation.

Summary and Conclusions

1. A brief discussion of some problems which should be considered by one interested in research in hypertension has been given. Etiologic and therapeutic aspects have been included.
2. A brief history of the discovery and development of the main pharmacologic actions of nitrites and organic nitrates has been presented. The question of the obligatory reduction of nitrates to nitrite before vasodilatation is produced has been reviewed. Some new viewpoints derived from various experiments reported have been discussed.
3. The main pharmacologic actions of a series of homologous organic nitrates have been presented. The sodium salt and the alkyl esters of the nitrate of glycollic acid from methyl to decyl, and the myristyl ester were studied. The salt is very weak, but has very low acute toxicity and can be injected in large amounts. After initial marked depressor responses, the blood pressure finally becomes constant at a level which is about 80 to 90 per cent of pre-injection level. Further injections have little or no effect. Blockade of vasoconstrictor outflow greatly augments the depressor action of this salt.
4. The alkyl esters of glycollate nitrate are considerably more potent than the salt, but far inferior to mannitol hexanitrate on a molar basis. On a "per nitrate group"

basis the latter is still several times more potent than the most potent member of the glycollate esters. This superiority is probably by the greater oil over water coefficient and greater ease of reduction by cellular systems.

5. A method for the bioassay of the relative potency of vasodilators of certain characteristics is described and many of the glycollate esters were assayed. The method should be very useful if the compounds in appropriate doses produce reproducible depressor effects of short duration with disappearance of the agents from the blood stream in reasonably short periods of time. The glycollates showed increasing potency with ascension of the alkyl ester series. A decrease in water solubility and thus in oil over water coefficients accompanies the ascent. The latter probably accounts for potency relationships. n-Heptyl glycollate nitrate is the most potent of the normal chain esters. The two iso- esters studied, the isobutyl and the isoamyl, were usually potent for their solubility, and were equivelent to the n-heptyl homolog in potency. None of these esters seems to offer therapeutic promise.

6. The activity of alkyl glycollate nitrates after treatment with strong alkali has been described. In several ways it resembles the actions of sodium nitrite. It may represent an easily hydrolyzed form of the nitrate.

It holds forth no promise as a hypotensive agent.

7. Several other hydroxy acid nitrates have been briefly studied for depressor potency. The sodium salt of saccharic acid dinitrate showed potentialities as long acting vasodilator. It was inadequately studied.

8. A few cellular functions were studied in regard to the possible mechanism of action of sodium nitrite as a muscle depressant. Adenosinetriphosphatase activity in smooth and skeletal muscle was found to be unaffected by reasonable and by excessive concentrations of nitrite. Oxygen consumption of tissue slices was found to be depressed only by very high concentrations of the salt. In concentration attainable by usual depressor doses in vivo no effects were produced. Cytochrome oxidase and reductase of brain tissue were unaffected by sodium nitrite. It is felt that further search along these lines is not warranted. The usefulness of the nitrites and nitrates is too limited, and the investigation is too involved.

9. As the chemistry of smooth muscle function is elucidated in the future, certain reactions may become known which will be amenable to control by drugs. If differences are found between arteriolar muscle cells and other smooth musculature, and a specific depression of activity can be effected in the former, that inhibitory mechanism may offer a valuable approach in the chemotherapy

of hypertension. There will still remain problems which may interdict the use of such an approach. Many functions of the body are disturbed by alterations in blood pressure. They must be studied when a vasodilator is being recommended for clinical use. If hypertension is frequently caused by psychosomatic factors, its prevention is not foreseen on any adequate scale. If it is humoral, greater optimism in prevention and treatment is justified. In the meantime, judicious use of nitrites and nitrates is occasionally justified. The further study of their possibilities is therefore warranted. If their usefulness is to be increased, it will probably be in the field of water soluble compounds.

SELECTED BIBLIOGRAPHY

1. Amberson, W. R.: J. Biol. Chem., In press.

2. Atkinson, G. A.: J. Anat. and Physiol., 22:225, 351, 1887.

3. Austin, J. H., and Drabkin, D. L.: J. Biol. Chem., 112:67, 1935.

4. Balard, M.: Ann. Chim. et Phys., 12:294, 1844.

5. Bellamy, W. D., and Klimek, J. W.: J. Bact., 55:153, 1948.

6. Bernheim: Pflüger's Arch. ges. Physiol., 8:253, 1874.

7. Bradbury, J. B.: Brit. Med. J., 2:1213, 1895.

8. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M.: Rev. soc. argintina biol., 15:420, 1939.

9. Brunton, T. L.: J. Anat. and Physiol., 5:92, 1870.

10. Brunton, T. L.: Lectures on the Action of Medicines. The Macmillan Co., New York (1897).

11. Cagnoli, M.: Jahresb. u. d. Fortschr. d. Thier-Chem., 15:92, 1885.

12. Cash, J. T., and Dunstan, W. R.: Phil. Trans. Roy. Soc. Lond. Series B, 184:505, 1894.

13. Coller, F. A., Campbell, K. N., Berry, R. E. L., Sutler, M. R., and others: Ann. Surg., 125:729, 1947.

14. Cox, W. W., and Wendel, W. B.: J. Biol. Chem., 143:331, 1942.

15. Crandall, L. A., Jr.: J. Pharmacol. and Exp. Therap., 48:127, 1933.

16. Crandall, L. A., Jr., Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W.: J. Pharmacol. and Exp. Therap., 37:283, 1929.

17. Dexter, L., and Haynes, F. W.: Proc. Soc. Exp. Biol. and Med., 55:288, 1944.

18. Dixon, M.: Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes, 2nd ed. The University Press, Cambridge (1943).

19. Drabkin, D. L.: Federation Proceedings, 7:483, 1948.

20. Drabkin, D. L., and Schmidt, C. F.: J. Biol. Chem., 157:69, 1945.

21. Dubois, K. P., and Potter, V. R.: J. Biol. Chem., 150:185, 1943.

22. Dunstan, W. R.: Pharm. J. Trans., Series 3, 19:485, 1888.

23. Evelyn, K. A., and Malloy, H. T.: J. Biol. Chem., 126:655, 1938.

24. Field, A. G.: Med. Times and Gaz., 16:291, 1858.

25. Field, C. E.: Lancet, 2:848, 1944.

26. Filehne: Arch. f. Anat. u. Physiol., 79:384, 1879.

27. Fishberg, A. M.: J. Am. Med. Assoc., 137:670, 1948.

28. Fiske, C. H., and Subbarow, Y.: J. Biol. Chem., 66: 375, 1925.

29. Forman, S. E., Carr, C. J., and Krantz, J. C., Jr.: J. Am. Pharm. Assoc., Sci. Ed., 30:132, 1941.

30. Freedberg, A. S., Spiegel, E. D., and Riseman, E. D.: Am. Heart J., 22:519, 1941.

31. Gamgee, A.: Proc. Roy. Soc. Edinburgh, 6:109, 1867, and Philosophical Transactions Roy. Soc. Lond., 158:589, 1868.

32. Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W.: J. Exp. Med., 59:347, 1934.

33. Goldring, W., and Chassis, H.: Hypertension and Hypertensive Disease. The Commonwealth Fund, New York (1944).

34. Guthrie, F.: J. Chem. Soc. Lond., 11:245, 1859.

35. Gutman, H. R., Jandorf, B. J., and Bodansky, O.: J. Biol. Chem., 169:145, 1947.

36. Haimovici, H., and Medinets, H. E.: Proc. Soc. Exp. Biol. and Med., 67:163, 1948.

37. Hay, M.: Practitioner, 30:179, 1883.

38. Ibid., p. 321.

39. Ibid., p. 422.

40. Hering, C.: Amerik. Arzneiprüfungen. C. F. Winter, Leipzig and Heidelberg (1857).

41. Herrman, R. F., Leake, C. D., Loevenhart, A. S., and Muehlberger, C. W.: J. Pharmacol. and Exp. Therap., 27:259, 1926.

42. Iwamoto, H. K., and Harman, J. B.: To be published.

43. Kielley, W. W., and Meyerhof, O.: J. Biol. Chem., 174:387, 1948.

44. Krantz, J. C., Jr., Carr, C. J., and Forman, S. E.: J. Pharmacol. and Exp. Therap., 64:298, 1938.

45. Ibid., p. 302.

46. Krantz, J. C., Jr., Carr, C. J., and Forman, S. E.: Proc. Soc. Exp. Biol. and Med., 42:472, 1939.

47. Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Cone, N.: J. Pharmacol. and Exp. Therap., 70:323, 1940.

48. Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Ellis, F. W.: J. Pharmacol. and Exp. Therap., 67:187, 1939.

49. Ibid., p. 191.

50. Leech, D. J.: Brit. Med. J., 1:1305, 1893.

51. Leech, D. J.: Brit. Med. J., 2:4, 1893.

52. Marshall, C. R.: J. Physiol., 22:1, 1897.

53. Marshall, C. R.: VIII Int. Congr. Appl. Chem., 19:211, 1912.

54. Marshall, C. R.: J. Pharmacol. and Exp. Therap. 83:106, 1945.

55. Matthew, E.: Quart. J. Med., 2:261, 1909.

56. Oberst, F. W. and Snyder, F. H.: J. Pharmacol. and Exp. Therap., 93:444, 1948.

57. Oberst, F. W., and Snyder, F. H.: To be published.

58. Page, I. H., and Corcoran, A. C.: Arterial Hypertension. The Yearbook Publishers Inc., Chicago (1946).

59. Page, I. H., and Helmer, O. M.: J. Exp. Med., 71:29, 1940.

60. Pelikan, E.: Beitr. z. Gerichtlich. Med., pp. 99-117, H. Goldstein, Wurtzburg (1858).

61. Price, W. H., and Cori, C. F.: J. Biol. Chem., 162: 393, 1946.

62. Rath, M. M.: Doctor of Philosophy Thesis, University of Maryland, 1942.

63. Rath, M., and Krantz, J. C., Jr.: J. Pharmacol. and Exp. Therap., 76:27, 1942.

64. Ibid., p. 33.

65. Reichert and Weir-Mitchell: Am. J. Med. Sci., 80:158, 1880.

66. Richardson, B.: Brit. and For. Med.-Chir. Rev. Lond., 40:259, 1867.

67. Stieglitz, E. J.: J. Am. Med. Assoc., 116:1383, 1941.

68. Snyder, F. H., Klahm, G. E., and Oberst, F. W.: To be published.

69. Snyder, F. H., and Oberst, F. W.: To be published.

70. Taylor, J. F., and Morgan, V. E.: J. Biol. Chem., 144:15, 1942.

71. Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O.: J. Biol. Chem., 166:121, 1946.

72. Von Oettingen, W. F.: Nat. Inst. Health Bull., 186: 1946.

73. Warburg, O., Kubowitz, F., and Christian, W.: Biochem. Z., 221:494, 1930.

74. Wendel, W. B.: J. Biol. Chem., 102:373, 1933.

75. Wood, H. C.: Am. J. Med. Sci., 61:422, 1871.

APPENDIX

TABLE 18

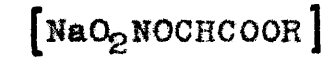
Depressor response of nitrates of glycollic acid esters

(Krantz, Carr, Forman and Cone, 1940)

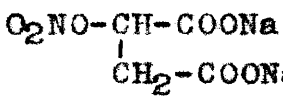
Name of Compound	Formula	Effective Depressor Molar Concentration	Oil/water Coefficient
Nitrate of sodium glycollate	$CH_2(NO_3).COO.Na$	0.10	0.9
Nitrate of ethyl glycollate	$.C_2H_5$	0.013	17
Nitrate of propyl glycollate	$.C_3H_7$	0.008	24
Nitrate of butyl glycollate	$.C_4H_9$	0.003	108
Nitrate of heptyl glycollate	$.C_7H_{15}$	0.001	142

TABLE 19

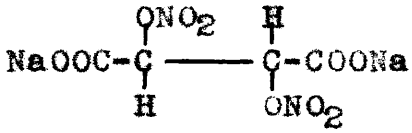
STRUCTURAL FORMULAS OF NITRATES USED IN THIS STUDY



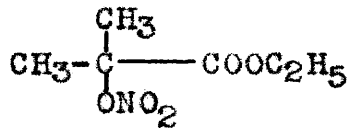
Sodium Alkyl Glycollate Nitrate



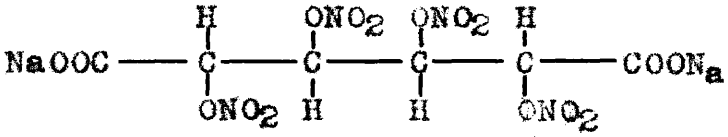
l-Disodium Malate Nitrate



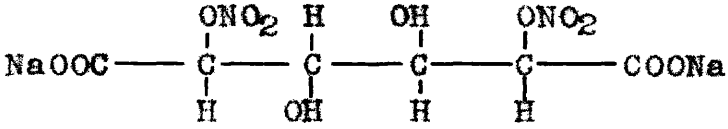
d-Disodium Tartrate Dinitrate



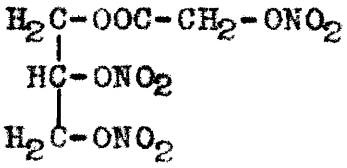
Ethyl Isobutyrate alpha-Nitrate



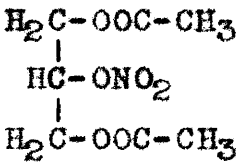
Disodium Mucate Tetranitrate



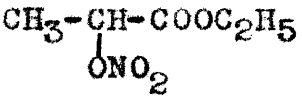
Disodium Saccharate Dinitrate



1-Glycollyl Glycerol Trinitrate



1,3-Diacetyl Glycerol-2-Nitrate



dl-Ethyl Lactate Nitrate

TABLE 20

Concentration of alcohol used as solvent in assays of alkyl glycollate nitrates

Alkyl group	Molarity	Per cent alcohol
Methyl	0.01	0
Ethyl	0.01	0
n-Propyl	0.01	5
Isopropyl	0.01	5
n-Butyl	0.01	21
sec-Butyl	0.01	17
Isobutyl	0.010	25
(Standard)	0.009	23.5
	0.008	20
	0.007	17.5
	0.006	15
	0.005	12.5
	0.004	10
	0.003	7.5
	0.0026	6.5
	0.002	5
	0.001	2.5
n-Amyl	0.01	35
Isoamyl	0.01	45
n-Hexyl	0.01	42
n-Heptyl	0.01	46
n-Octyl	0.01	58

TABLE 21

Assay of n-propyl glycollate nitrate
Dog No. 81 1/8 cc. per kilogram

Effect on blood pressure - per cent fall			
n-Propyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.003M.	0.004M.	0.01M.
13.4	7.0	12.7	28.8
11.6		10.3	
10.3		10.3	
Average 11.8		11.1	
Isobutyl Rating: 0.4			

TABLE 22

Assay of isopropyl glycollate nitrate
Dog No. 85 0.5 cc. per kilogram

Effect on blood pressure - per cent fall			
Isopropyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.003M.	0.004M.	0.005M.
6.7	3.6	6.7	8.3
6.7		6.7	7.0
6.9		6.9	
Average 6.8		6.8	7.8
Isobutyl Rating: 0.4			

TABLE 23

Assay of n-butyl glycollate nitrate
Dog No. 85 0.5 cc. per kilogram

Effect on blood pressure - per cent fall			
n-Butyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.003M.	0.004M.	0.005M.
6.7	3.6	7.6	8.5
6.8		7.0	7.0
5.3		6.7	
5.5		6.6	
7.1		6.7	
4.2		6.7	
6.7		6.9	
6.6			
Average 6.1		6.8	7.8
	Isobutyl Rating:	0.4	

TABLE 24

Assay of sec-butyl glycollate nitrate
Dog No. 78 0.25 cc. per kilogram

Effect on blood pressure - per cent fall			
sec-Butyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.0026M.	0.003M.	0.004M.
10.0	9.9	13.7	15.0
12.0		12.5	
13.4		12.7	
9.8		11.3	
13.4		10.5	
Average 11.7		12.1	
	Isobutyl Rating:	0.3	

TABLE 25

Assay of n-amyl glycollate nitrate
Dog No. 81B 1/6 cc. per kilogram

Effect on blood pressure - per cent fall				
n-Amyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.005M.	0.006M.	0.007M.	0.008M.
10.8	7.3	10.0	9.6	12.7
9.6	5.5	10.9	9.4	
8.6			8.3	
Average 9.7	6.4	10.5	9.1	
Isobutyl Rating: 0.6 to 0.7				

TABLE 26

Assay of isoamyl glycollate nitrate
Dog No. 82 1/12 cc. per kilogram

Effect on blood pressure - per cent fall		
Isoamyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.008M.	0.01M.
10.9	8.3	10.1
10.6	8.9	10.4
11.4		14.3
10.6		
Average 11.6	8.6	11.6
Isobutyl Rating: 1.0		

TABLE 27

Assay of n-hexyl glycollate nitrate
Dog No. 83 0.5 cc. per kilogram

Effect on blood pressure - per cent fall		
n-Hexyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.005M.	Isobutyl glycollate nitrate 0.006M.
7.4	5.8	8.8
7.2	3.2	6.7
7.4		7.3
7.3		5.7
4.8		5.7
Average 6.8	4.5	6.8
Isobutyl Rating: 0.6		

TABLE 28

Assay of n-heptyl glycollate nitrate
Dog No. 81 1/8 cc. per kilogram

Effect on blood pressure - per cent fall	
n-Heptyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.01M.
27.4	30.6
29.8	29.2
27.7	26.7
Average 28.3	28.8
Isobutyl Rating: 1.0	

TABLE 29

Assay of n-octyl glycollate nitrate
Dog No. 81 1/8 cc. per kilogram

Effect on blood pressure - per cent fall			
n-Octyl glycollate nitrate 0.01M.	Isobutyl glycollate nitrate 0.006M.	0.007M.	0.01M.
10.5	10.6	11.9	16.0
10.6	13.3	11.7	
13.3	13.3		
14.5	11.0		
Average 12.2	12.0	11.7	
	Isobutyl Rating: 0.6 to 0.7		