The Comparative Physiological Action of some Derivatives of Guanidine.

Thesis by

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Introduction.

Guanidine, methylguanidine and asymmetric dimethylguanidine are of extended biological interest. Guanidine itself has been isolated as a nitrogenous constituent of some plants,[1,2] while methyl and dimethyl guanidine probably play a part in intermediary nitrogen metabolism and have been isolated as physiological constituents in the urine of the dog, horse and man.[3,4,5]

Pathologically these compounds have considerable interest from many points of view. Koch [6] found methylguanidine in the urine of parathyroidectomized dogs and later reported the presence of guanidine, symmetric and asymmetric dimethylguanidine, choline, neurine and histamine. [7] Henderson [8] reports that with such dogs there is a decreased muscular content of substances containing the guanidine complex. Burns and Sharpe [9] carefully determined the guanidine and methylguanidine in the blood of animals after parathyroidectomy and found a marked increase of these substances, together with an increase of these bases in the urine, as had been reported by Koch. Paton and Findlay [10] showed that the symptoms of tetania parathyreopriva are identical with those produced by the administration of guanidine and methylguanidine, and Watanabe [11] has shown that the injection of guanidine causes a hypoglucemia, calcium decrease in the blood, phosphate retention and an acidosis similar to the metabolic changes occuring after parathyroidectomy. Burns and Sharpe [9] found an

increased content of guanidine and methylguanidine in the urine of children with idiopathic tetany and Findlay and Sharpe [12] and Nattrass and Sharpe [13] found an increased excretion of dimethylguanidine. Methylguanidine has been isolated from the faeces of children with idiopathic tetany.

Major [15] believes that there is a possible relationship between guanidine metabolism and hypertension in man. Fairly large quantities of methylguanidine are reported to occur in the urine of animals killed by anaphylactic shock or by burning, according to Heyde [16,17] and it has been suggested that the poison producing shock may be the methylguanidine elaborated by the toxicogenic destruction of protein. Heyde reported [16] that methylguanidine poisoning in guinea-pigs and rabbits showed a marked similarity to anaphylactic shock, but Führer[18] using rabbits and Loewitt [19] using guinea-pigs were not able to observe any marked lung action when using a synthetic methylguanidine.

Guanidine and especially methylguanidine have been shown to be active in decreasing the poisonous action of protein split-products by weichardt and Schwenk.[20] Similar to this is the observation of Burns that methylguanidine protects sensitized animals from toxic doses of antigenic protein. The possible relationship of the guanidines to epilepsy, epidemic encephalitis and various protein intoxications has been discussed in the literature.

Because of the widespread interest regarding the physiological activity of guanidine, its methyl and asymmetric dimethyl derivatives, it seemed that a study ~~between~~ of the relation between chemical constitution and certain physiological actions of several guanidine derivatives would be desirable. Pharmacological studies have been

made by previous workers with the following derivatives [22,23]; guanidine, methylguanidine, asymmetric dimethylguanidine, dicyandiamidine, glycocyamine, creatine and agmatine . These studies in the case of guanidine, methylguanidine and dimethylguanidine have fairly completely covered their general physiological action, but little attempt has been made to carefully compare their relative physiological activity. For the study of the derivatives prepared in this work it was decided that careful observations of their blood pressure and respiratory effects, together with toxicity determinations would offer the best comparisons of their relative physiological activity.

The glucemic effect of these derivatives was also investigated as it seemed probable that a variation in chemical constitution might produce a substance having an hypoglucemic activity much greater than that of guanidine itself. [11] Such a fact would be of considerable importance with regard to speculations concerning a possible chemical constitution of insulin.

I here wish to express my most sincere thanks to Dr. E. M. K. Geiling of the Johns Hopkins Medical School for his help and encouragement in performing parts of this work. Further thanks are due to Dr. P. J. Hanzlik of the Stanford University Medical School as well asto Dr. Geiling for their advice in preparing this paper.

For convenience the work is reported in four parts.

I. Preparation and Identification of some Guanidine Derivatives.

II. Blood Pressure and Respiratory Effect of some Guanidine Derivatives.

III. Toxicity of some Guanidine Derivatives.

IV. Glucemic Effect of some Guanidine Derivatives.

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I. Preparation and Identification of some Guanidine Derivatives.

The methods of preparation and data for characterization of a large number of the simpler guanidine drivatives are available in the literature. In general the methods of preparation are quite satisfactorily developed. The work here reported describes the preparation of two new guanidine derivatives as hydrochlorides, together with a description of the methods used in preparing the previously known guanidine derivatives that were used in this work. Picrates of all the guanidine derivatives were prepared to serve as means of identifying these bases.

Experimental.

Guanidine. Guanidine carbonate was obtained from the Eastman Kodak Company and converted into the sulfate and the hydrochloride. The sulfate was recrystallized from hot methyl alcohol, washed and then dried at 105°C. The chloride was recrystallized from hot ethyl alcohol and dried. The dried sample melts at 184°C.

Cl calcd. for $CH_5N_3.HCl$ - 37.12% Cl found - 37.01%, 37.07%

The picrate of this base was prepared and recrystallized from hot water. It did not melt up to 260°C. but decomposed at this temperature in 15 minutes. In the literature [1] it is stated that this picrate blackens at 280°C and decomposes at 311-315°C.

Methylguanidine. Methylguanidine sulfate was obtained from the Eastman Kodak Company and a part was converted into the hydrochloride using barium chloride and avoiding the slightest excess. The sulfate as obtained had been obtained from methyl iso-thiourea sulfate and methylamine in the manner described by Phillips and Clarke[2]. The sulfate was recrystallized from hot methyl alcohol,

washedand then dried at 105'C. The chloride was recrystallized from hot ethyl alcohol and dried. The dried sample melts at 138-142'C.

Cl calcd. for $C_2H_7N_3.HCl$ - 32.37% Cl found - 32.15%, 32.21% The picrate of this base was prepared and recrystallized from hot water, giving mixed orange prisms and yellow needles. It was possible to recrystallize the yellow needles without change. Both forms had the same melting point 199-200'C. [2,3] The fact that methylguanidine picrate exists in two forms has been reported by Guletwitsch. [4]

Asymmetric Dimethylguanidine. Dimethylguanidine sulfate was obtained from the Eastman Kodak Company anda part converted into the hydrochloride using barium chloride and avoiding the slightest excess. The sulfate as obtained hd been prepared frmm methyl iso-thiourea sulfate and dimethylamine in the manner described by Phillips and Clarke[2]. The sulfate was recrystallized from hot methyl alcohol, washed and then dried at 105'C. The chloride was recrystallized from hot ethyl alchhol and dried. The dried sample melts at 120-123'C.

Cl calcd. for $C_3H_9N_3.HCl$ - 28.69% Cl found - 28.73%, 28.65% The picrate of this base wase prepared and recrystallized from hot water giving a fine yellow crystal powder melting at 224'C. which is the melting point given by Wheeler and Jamieson. [5]

Ethylguanidine. Ethylguanidine sulfate was prepared from ethylamine and methyl iso-thiourea sulfate following the general method of Phillips and Clarke[2]. 17.5 g. of methyl iso-thiourea sulfate and 20 g. of a 33% aqueous solution of ethylamine gave a yield of 12.2 g. ethylguanidine sulfate. This is 85% of theoretical.

A part of the sulfate was converted into the hydrochloride using barium chloride and avoiding any excess. Ethylguanidine and its salts have not been previously described in the literature.

The sulfate was recrystallized from hot methyl alcohol, 5.0 g. being dissolved in 100 cc and 3.4 g. crystallized out. The hydrochloride was recrystallized from hot ethyl alcohol and dried at 105°C. The dried sample melts at 120-123°C., the same as the hydrochloride of dimethylguanidine.

Cl calcd. for $C_3H_9N_3.HCl$ - 28.69% Cl found - 28.23%, 28.88%

The picrate of this base, recrystallized from hot water in orange needles, melts at 176°C.

It may be mentioned here that two attempts to prepare asymmetric diethylguanidine salts using the same procedure as above were not succesful. It was not possible to induce crystallization of the product obtained.

Ethanolguanidine. Ethanolguanidine sulfate was prepared from ethanolamine and methyl iso-thiourea sulfate following the general method of Phillips and Clarke[2]. 16. g. of methyl iso-thiourea sulfate and 8. g. of ethanolamine were used, giving 7.0 g. of ethanolguanidine sulfate. This yield is 47% of theoretical. A part of the sulfate was converted into the hydrochloride using barium chloride and avoiding an excess. Ethanolguanidine and its salts have not bee previously prepared.

The sulfate was recrystallized from hot methyl alcohol, 5.0 g. baing dissolved in 100 cc and 2.7 g. crystallized out. The hydrochloride was recrystallized from hot ethyl alcohol then dessicated over $CaCl_2$. The dried sample melts at 85-95°C.

Cl calcd. for $C_3H_9ON_3.HCl$ - 25.40% Cl found - 25.21%, 25.14%

The picrate of this base, recrystallized from hot water in yellow needles, melts at 148°C.

Two attempts to prepare asymmetric diethanolguanidine using diethanolamine and the same procedure as above were not succesful. It was not possible to induce crystallization of the product obtained either as the sulfate or as a hydrochloride.

Acetylguanidine. Guanidine hydrochloride was acetylated with acetyl chloride following the method described by Korndörfer.[6] 10. g. of guanidine hydrochloride and 10. g of acetyl chloride were heated together in a cloded tude at 100°C. for one hour. The resulting product was crystallized from hot ethyl alcohol. The hydrochloride was recrystallized from hot ethyl alcohol and dried. The dessicated sample melted at 126-128°C. while Korndörfer reported a melting point of 140-142°C. This difference is large and its cause is not evident for the melting point determination was made on a sample dried for one week over sulfuric acid and which gave the following analytical figure.

Cl calcd. for $C_3H_7ON_3.HCl$ - 25.78% Cl found - 25.68%

The picrate of this base, recrystallized from hot water in glistening yellow needles, melted with decomposition at 225-230°C.

Aminoguanidine. The bicarbonate of this base was prepared from the reduction product of nitroguanidine when treated with equivalent amounts of acetic aaid and zinc as described by Thiele.[7] 20. g. of nitroguanidine, 70 g. of zinc dust and 12 cc of acetic acid were used. Treatment of the resulting solution with sodium bicarbonate yielded 12 g. of aminoguanidine bicarbonate. The bicarbonate was converted into the chloride. The hydrochloride was recrystallized from hot

dilute ethyl alcohol. The dried hydrochloride melted at 162-163°C. Thiele[7] gives the melting point as 163°C.

Cl calcd. for $CH_6N_4.HCl$ - 32.07% Cl found - 32.51%

The picrate of this base, recrystallized from hot water melted at 205°C.

Phenylguanidine. Several attempts to prepare this compound by the desulfurization of phenylthiourea in the presence of ammonia, either with litharge or lead hydroxide, were unsuccesful. The desulfurization appeared to follow the same course whether ammonia was present or not, chiefly to produce triphenyl-iso-melamin which is formed from the phenyl cyanamid produced as the first step in the desulfurization process. Feuerlein [8] reported that phenylguanidine could be prepared by this method but McKee [9] reported that Feuerlein probably never isolated phenylguanidine.

The best method of preparing this compound appears to be that of McKee [9], starting with aniline hydrochloride and cyanamide. Considerable difficulty was encountered in preparing fairly large amounts of unpolymerized cyanamide and attempts to use the polymer dicyandiamidine were unsuccesful, yielding only phenyl biguanid hydrochloride.

Aniline hydrochloride and urea can be condensed to form phenylurea by boiling together in solution and it was thought that a similar reaction might be carried out with guanidine to form the phenyl derivative. Aniline hydrochloride and guanidine hydrochloride were heated together in absolute alcohol but no reaction occured.

Symmetric Diphenylguanidine. The free base was obtained from the Eastman Kodak Company and had probably been prepared by the desulfurization of thiocarbanilide with lead oxide. Diphenylguanidine is extensively used as a vulcanization accelerator. The free base as obtained melted at 146-148°C. The hydrochloride was prepared by

dissolving the base in an alcohol- ether mixture and passing in anhydrous hydrogen chloride. The precipitated mass becomes crystalline on standing. This hydrochloride has been previously described in the literature as a non-crystallizable gummy mass. The hydrochloride is recrystallized from hot ethyl alcohol and when dried melts at 130-132'C.

Cl calcd. for $C_{13}H_{13}N_3.HCl$ - 14.32% Cl found - 14.20%

The picrate crystallizes from hot water in fine yellow needles, which melt at 164'C.

Triphenylguanidine. The free base was obtained from the Eastman Kodak Company and it was probably obtained from a commercial product prepared by heating thiocarbanilid with aniline. The melting point of the free base was 143-144'C.[10] The hydrochloride was preparedbby treating the base with a slight excess of hydrochloric acid and crystallizing from hot ethyl alcohol. The recrystallized hydrochloride melts at 246-248'C.

Cl calcd. for $C_{19}H_{18}N_3.HCl$ - 10.95% Cl found - 10.64%

The picrate of this base crystallizes from hot water in fine yellow needles which melt at 181-182'C. The melting point of this picrate has been reported by Dains [11] as 180'C.

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II. Blood Pressure and Respiratory Effects of some Guanidine Derivatives.

Studies of the blood pressure effect of different substances have been much uded, especially in the adrenaline series, in the study of relationships between chemical constitution and physiological effect. The chief reasons for this are two, first, blood pressure experiments can be made semi-quantitativ in nature and second, a great deal of pharmacological study has been devoted to the mechanism of such effects, allowing interpretations of the observed effects.

Because of the considerable biological interest in guanidine, its methyl and dimethyl derivatives, a study has been made of the blood pressure effects of a number of guanidine derivatives. Combined with this, studies have been made of the respiratory effect of these same derivatives.

Blood pressure investigations with guanidine itself have been reported by Jordan,[1] Rothberger,[2] and quite recently by Major and Stephenson.[3] These investigations were carried out with cats and dogs, and observations of the respiratory effect were made in some instances. An effect of guanidine on the respiratory rate in rats has been reported by Klinger.[4] Blood pressure studies with methylguanidine have been made by Loewitt[5] using guinea-pigs, by Heyde[6] using rabbits and by Major and Stephenson[7] using dogs as the experimental animals. No observations other than those of Major and Stephenson [3] have been found in the literature as to the effects of asymmetric dimethyl-guanidine, while the effects of the other guanidine derivatives reported upon in this paper have not been previously studied.

The derivatives studied in this work are guanidines substituted

by methyl, ethyl, ethanol phenyl, acetyl or amino groups. All of the compounds were synthetically prepared and were used in a state of chemical purity as judged by the melting points and analyses of the hydrochlorides of the bases and the melting points of the picrates.

Experimental.

Rabbits weighing approximately 3 kg. were used throughout this work and the procedure and technic of performing the blood pressure and respiratory observations were standardized as carefully as possible. The animals were kept without food 16 to 24 hours previous to the experiment. Urethane (1.5 to 2.0 g. per kg.) in 20% aqueous solution was injected subcutaneously for anaesthesia, ether being generally used in small amount as an aid in inducing a complete anaesthesia during the operative procedure. The procedure in each experiment was as follows. The urethane was injected; 30 minutes later a small amount of ether was administered; at 40 minutes the insertion of a tracheal cannula and an arterial cannula into the left carotid was made and at one hour the blood pressure and respiratory tracings were begun. All solutions were injected intravenously into a marginal ear vein and the amounts reported are in terms of free base although the sulfate or hydrochloride was used in making up the solution.

Guanidine. Solutions of both the sulfate and the hydrochloride were used but no qualitative or quantitative differences in result were observed. Fig. 1. illustrates a typical response to guanidine in the rabbit. Immediately after injection there is a decrease in blood pressure followed in about 20 seconds by a rise that may persist for from 10 to 15 minutes before returning to normal. Usually, the

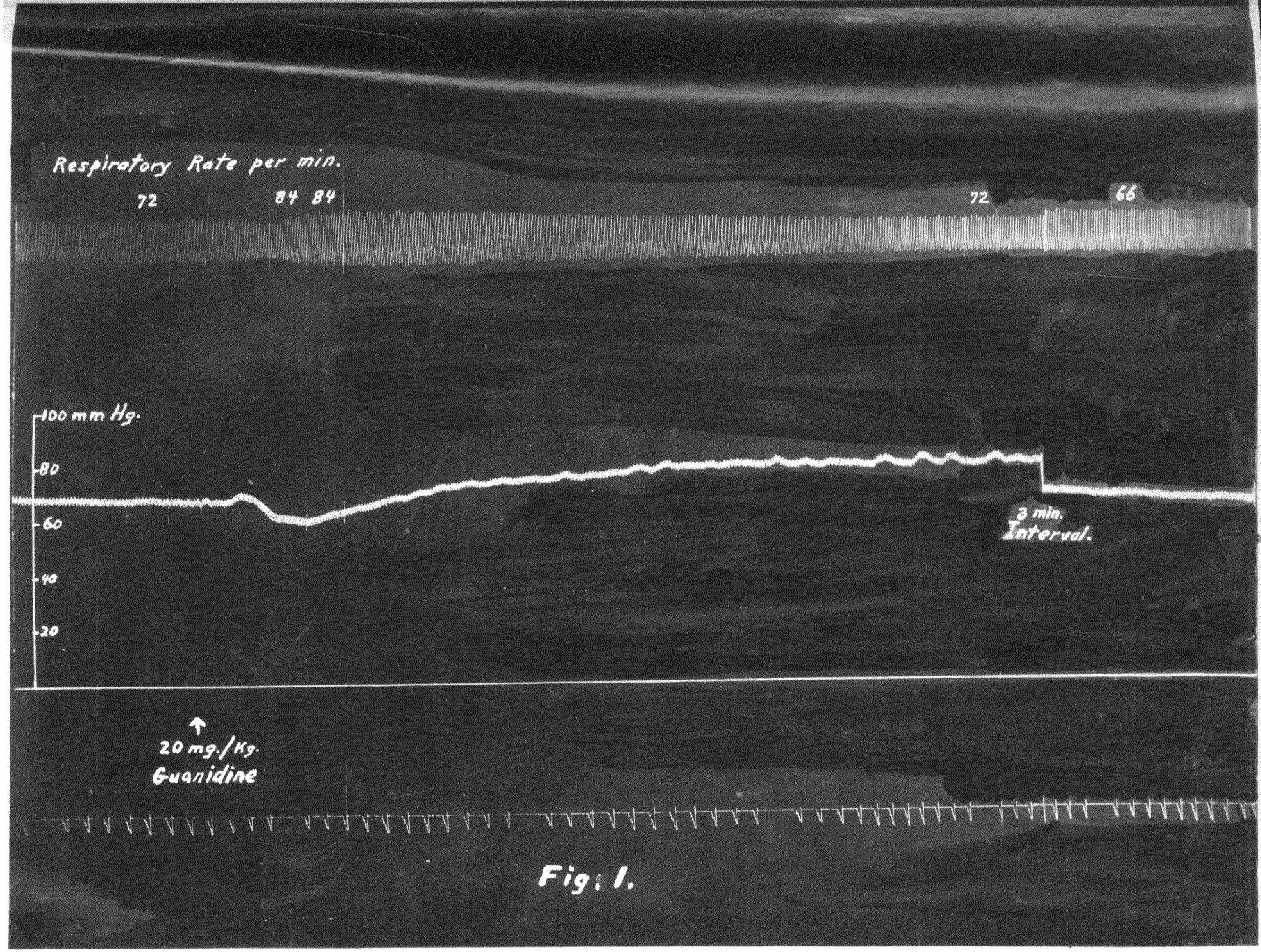
Respiratory Rate per min.
72
84 84
72
66
100 mm Hg.
80
60
40
20
3 min. Interval.
20 mg./Kg. Guanidine

Fig. 1.

return to normal takes place in from 6 to 7 minutes. The pressure changes vary considerably with different animals, a dosage of 10 mgm. of guanidine per kg. causing decreases in pressure of from 6 to 16 mm. of mercury, followed by increases in pressure of from 6 to 18 mm. of mercury, while the variation in response towards larger doses is even greater. The heart rate is not appreciably changed during the periods of pressure change, as is shown by the following record of Experiment XVII.

Experiment XVII. rabbit weighing 3.3 kg. Urethane anaesthesia.

Guanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Blood Pressure (mm.Hg)			Heart Rate per minute.		
	Normal	Min.	Max.	Normal	After injection.	
20	74	62	98	322	317	316
After 2 mgm. per kg. atropine sulfate.						
20	86	70	102	304	306	300

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute.		
	Normal	At Max. Amplitude	At Min. Amplitude
20	76	82	70
After 2 mgm. per kg. atropine sulfate.			
20	84	84	84

No change in the amplitude of the heart beat, as judged from the blood pressure records, occured in any of the experiments. With a few animals the preliminary fall in pressure was absent, only a gradual rise to maximum pressure occuring after injection. Atropinization appears to have no effect upon the circulatory response to guanidine indicating that the action of guanidine occurs independently of the vagal innervation of the heart and suggests that the fall of blood pressure is due to direct cardiac depression.

The respiration is stimulated by guanidine, causing an increase in amplitude accompanied by an increase in rate and usually followed by recovery. A complete recovery does not occur in some animals. Occasionally, as in Fig.1. the increase in amplitude is preceded by a short period of decreased amplitude although the rate is increased immediately after injection. Atropinization abolishes the change in respiratory rate caused by guanidine, although the increase in amplitude appears to be unaffected. (See Experiment XVII.)

Methylguanidine. Solutions of both the sulfate and the hydrochloride were used but no differences in result were observed. Fig. 2. shows the usual response to methylguanidine in the rabbit, the response being considerably greater than to guanidine. The preliminary fall of blood pressure immediately after injection is of shorter duration and less in magnitude than with guanidine. The increase in pressure is much the same, though it requires but about one fourth as much methylguanidine to produce equivalent effect. With large doses of methylguanidine the increase in pressure may be followed by a large and prolonged decrease in pressure. A few animals respond with a prolonged decrease in pressure followed by a slight increase in pressure, but Fig.2. can be considered as the typical effect of methylguanidine upon the blood pressure. The heart rate is decreased during the periods of blood pressure change, the effect being much the same regardless of the magnitude or type of blood pressure response. The magnitude of the effect is shown in the following record of Experiment XXXII.

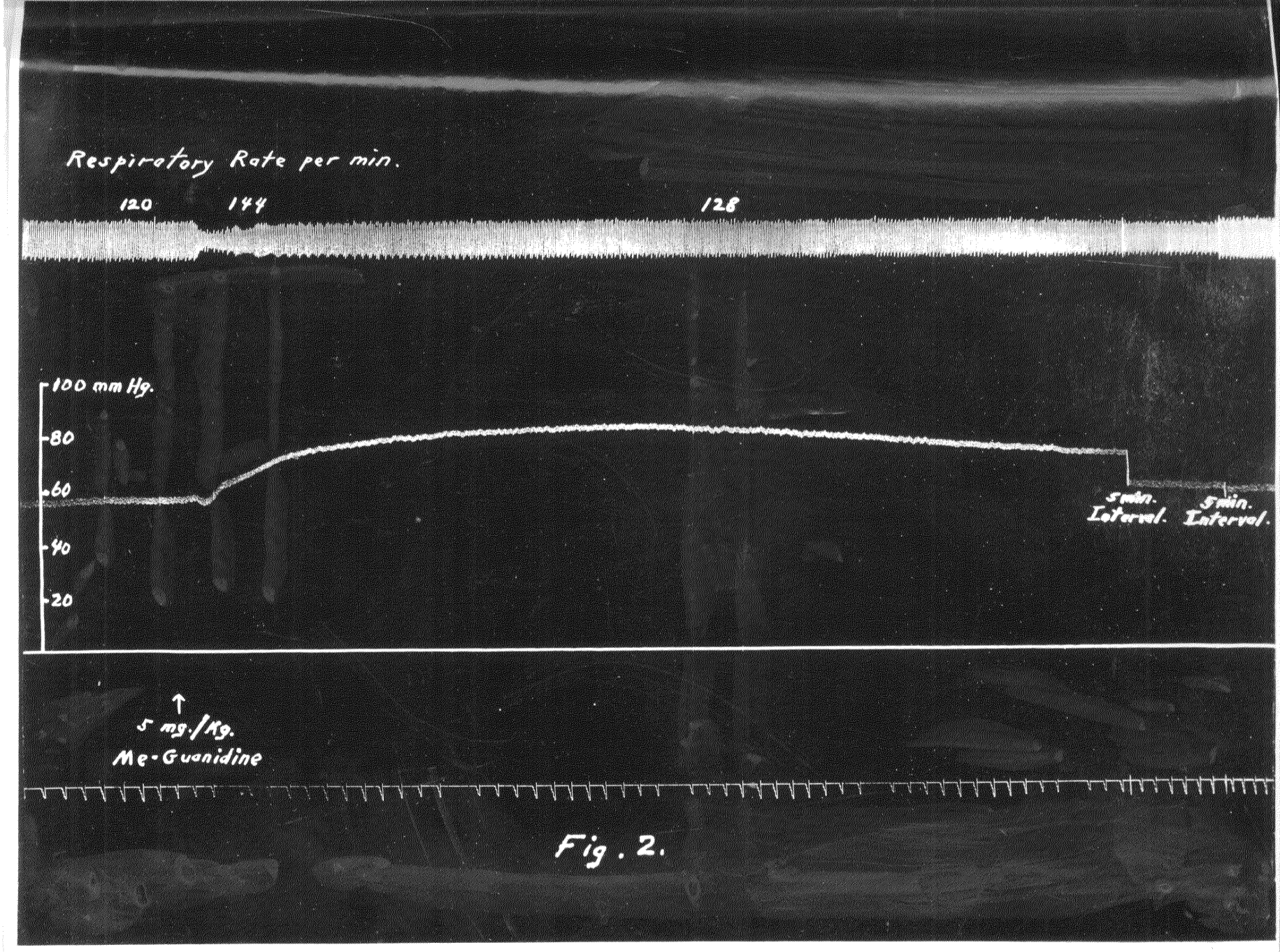
Respiratory Rate per min.
120
144
128
100 mm Hg.
80
60
40
20
5 min. Interval.
5 min. Interval.
5 mg./Kg.
Me-Guanidine

Fig. 2.

Experiment XXXII. rabbit weighing 2.95 kg. Urethane anaesthesia.

Methylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Min. Pressure	At Max. Pressure
5	304	284	306
After 2 mgm. per kg. atropine sulfate.			
5	304	288	300

Respiratory effect.

Dose in mg. of base per kg.	Respiratory Rate per minute. Normal	At Min. Amplitude	At Max, Amplitude
5	66	72	66
After 2 mgm. per kg. atropine sulfate.			
5	66	96	80

The amplitude of the heart beat appeared to be unaffected by the injection of methylguanidine as judged by the blood pressure tracing. Atropinization appears to have no effect on the circulatory response to methylguanidine, the blood pressure effects and the heart rate changes being the same after atropine as before.

The respiration is strongly affected by methylguanidine, causing a considerable decrease in amplitude accompanied by an increase in rate followed by a return of both amplitude and rate to normal. The increase in the respiratory rate after methylguanidine is much the same as the increase with guanidine even though much smaller doses are used (one-fourth). The increase in amplitude of respiration observed with guanidine was never observed when using methylguanidine. Atropinization causes the increase in respiratory rate following the injection of methylguanidine to become more marked although the amplitude changes remain the same.

Asymmetric Dimethylguanidine. Solutions of both the sulfate and the hydrochloride were used but no differences in results were observed. Fig. 3, shows the response to injection of asymmetric dimethylguanidine in the rabbit, the response being much more marked than to guanidine and practically the same as the response to methylguanidine. With some animals the blood pressure curve has an identical form as that of Fig..2. except that the double pressure maximum as shown in Fig. 3. usually appears. The haart rate is affected to a much greater extent and for a longer time than with the same dose of methylguanidine. The results of Experiment XXXI show the magnitude of the changes in heart rate following the injection of dimethylguanidine.

Experiment XXXI, rabbit weighing 3.3 kg. Urethane anaesthesia.

Asymmetric Dimethylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Min. Pressure	At Max. Pressure
5	312	256	264
After 2 mgm. per kg. atropine sulfate.			
5	296	252	256

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	At Min. Amplitude.	At Max. Amplitude
5	70	88	72
After 2 mgm. per kg. atropine sulfate.			
5	72	98	72

The amplitude of the heart beat increases shortly after the injection of dimethylguanidine. Atropinization appears to have no effect on the circulatory response to dimethylguanidine, the blood pressure and heart rate changes being the same after atropine as before. (See Experiment XXXI.) This suggests direct cardiac depression

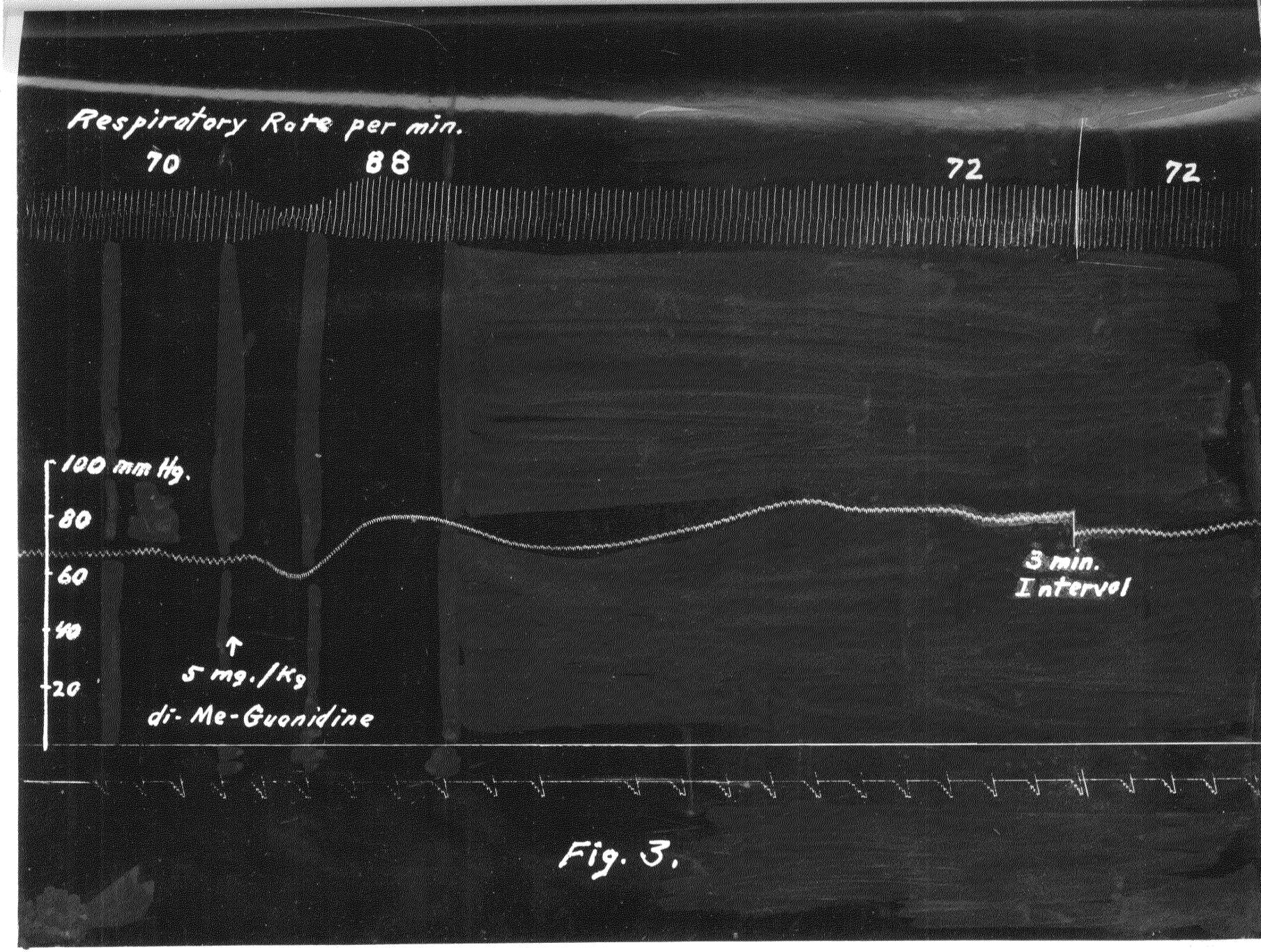
Respiratory Rate per min.
70
88
72
72
100 mm Hg.
80
60
40
20
5 mg./Kg
di-Me-Guonidine
3 min.
Interval

Fig. 3.

as the cause of the fall in blood pressure.

The respiration is markedly affected by dimethylguanidine, causing a marked decrease in amplitude accompanied by increase in rate and followed by a return to normal. The effect of methyl and dimethylguanidine are much the same. Atropinization causes the increases in respiratory rate following the injection of dimethylguanidine to become more marked although the amplitude changes remain the same.

Ethylguanidine. Fig. 4. shows the response to ethylguanidine in the rabbit. The response, a sharp rise to a maximum pressure with an abrupt decrease to a lower and prolonged pressure level is characteristic for this substance. Large doses of this substance may cause a fall in pressure especially when repeated or given after the injection of other guanidine compounds. Ethylguanidine has approximately the same intensity of effect as the methyl or dimethyl derivatives, equal doses producing equivalent changes in blood pressure. The heart rate is affected to about the same extent as with dimethylguanidine, although the effect seems to be less prolonged. The results of Experiment XX show the magnitude of the effect of ethylguanidine on the heart rate.

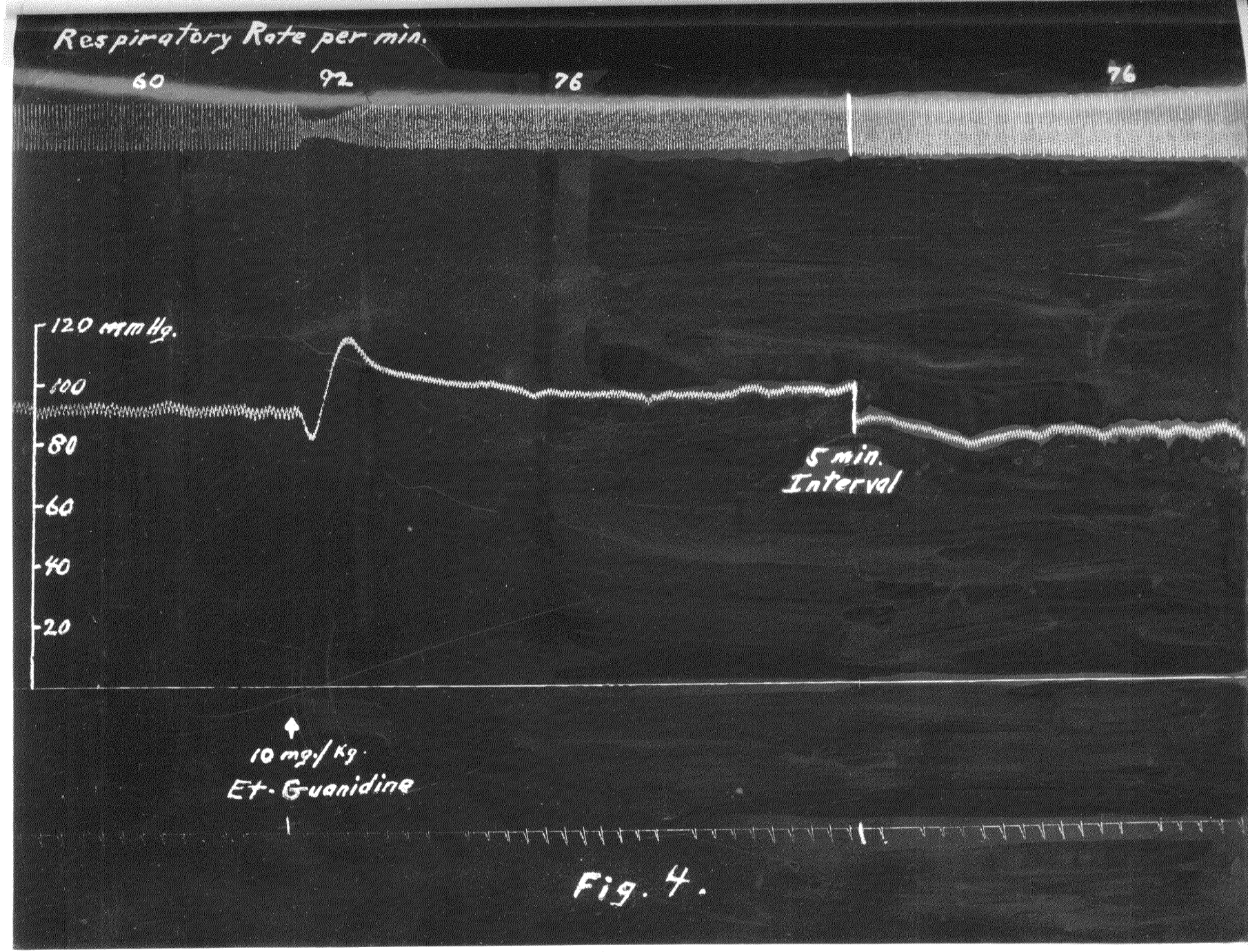
Respiratory Rate per min.
60
92
76
76
120 mm Hg.
100
80
60
40
20
5 min.
Interval
10 mg./Kg.
Et. Guanidine

Fig. 4.

Experiment XX. rabbit weighing 2.9 kg. Urethane anaesthesia.

Ethylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Max. Pressure	At 2 min.
10	348	180	348
After 2 mgm. per kg. atropine sulfate.			
10	348	138	336

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	At Min. Amplitude	At 2 min.	At 12 min.
10	168	180	204	156
After 2 mgm. per kg. atropine sulfate.				
10	84	126	102	86

The amplitude of the heart beat is much increased after the injection of ethylguanidine, paralelling the decrease in heart rate, suggesting marked cardiac depression. Atropinization appears to decrease the blood pressure response to ethylguanidine, together with a greater decrease in heart rate and a greater increase in amplitude of the heart beat. (See Experiment XX.)

The respiration is affected to about the same extent as with methyl and dimethyl guanidines, the amplitude being very markedly decreased immediately after the injection of the ethylguanidine. The decrease in amplitude is accompanied by an increase in rate as with other guanidine derivatives. Atropinization causes the increase in respiratory rate following the injection of ethylguanidine to become even greater, without any change in response with regard to the amplitude of respiration.

Ethanolguanidine. Fig. 5. shows the response to injection of ethanolguanidine in the rabbit. The response is less marked than with methyl or ethyl guanidines and there is a greater

tendency to cause a fall in blood pressure with large doses. Ethanolguanidine is only about one- half as active as the methyl and ethyl derivatives in the raising of the blood pressure and the effect on the heart rate is even less than in this ratio. The effect on the heart rate, as with ethylguanidine, is not prolonged and does not last more than two or three minutes. The results of Experiment XXIV show the magnitude of the effect of ethanolguanidine on the heart rate.

Experiment XXIV. rabbit. weighing 2.95 kg. Urethane anaesthesia.

Ethanolguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Min. Pressure	At Max. Pressure
5	300	270	294
After 2 mgm. per kg. atropine sulfate.			
5	300	276	300

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	At Min·Amplitude	At Max. Amplitude
5	40	48	42
After 2 mgm. per kg. atropine sulfate.			
5	48	68	60

The amplitude of the heart beat is but slightly increased after the injection of ethanolguanidine. The decrease in heart rate and the increase in amplitude of the beat are probably caused by the same effect of the guanidines and the lesser activity of ethanolguanidine is exhibited in bothe effects. Atropinization seems to have no effect on the circulatory response to ethanolguanidine, the blood pressure effects and the heart rate changes being unaffected.

The respiration is affected less with ethanolguanidine than with the methyl and ethyl derivatives, both as to changes in amplitude

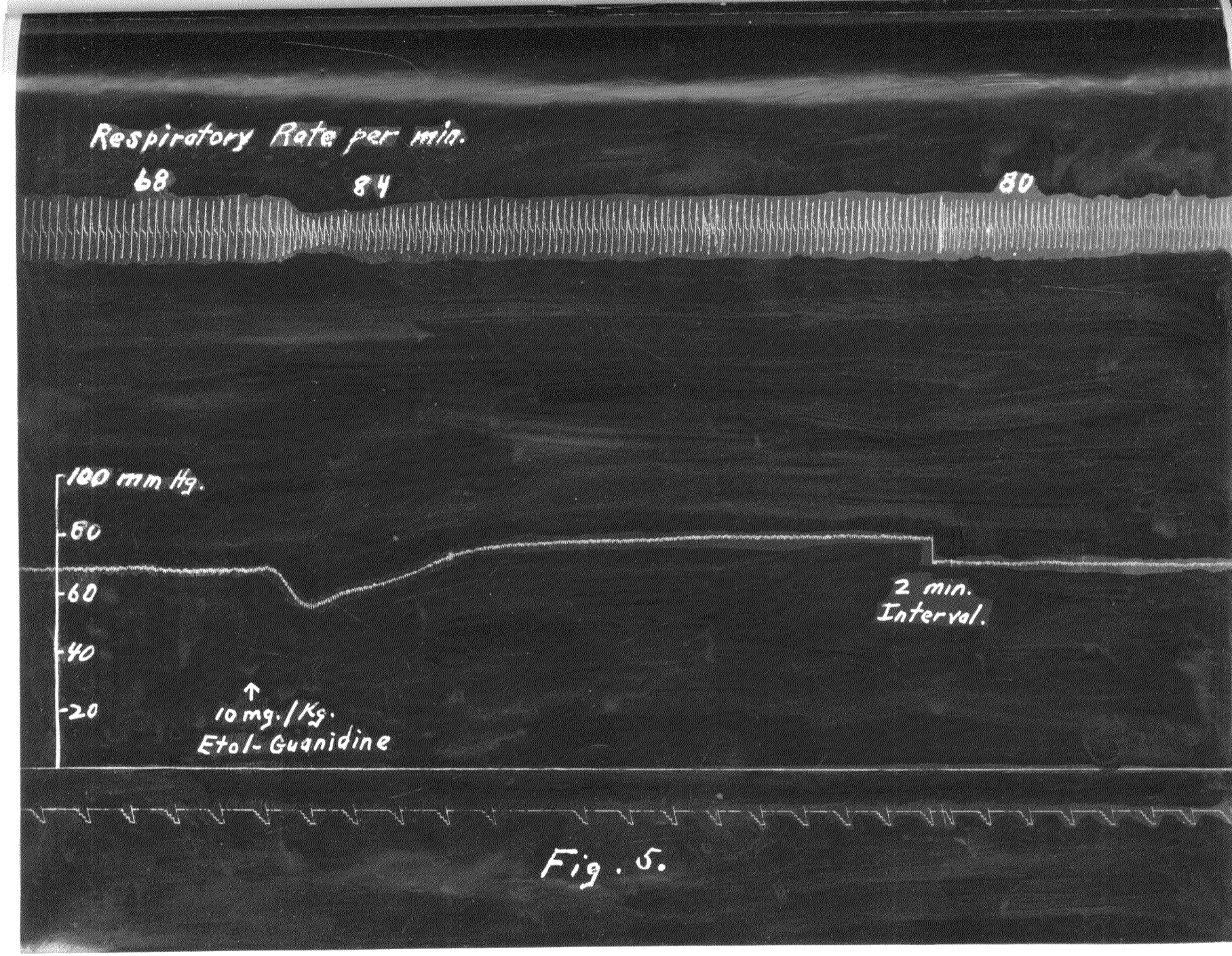
Respiratory Rate per min.
68
84
80
100 mm Hg.
60
60
40
20
10 mg./Kg.
Etol-Guanidine
2 min.
Interval.

Fig. 5.

and rate, although the qualitative effects are the same. Atropinization causes the increase in respiratory rate to become more marked.

Acetylguanidine. Fig. 6. shows the response to acetylguanidine in the rabbit, the response being many times more marked than to guanidine itself. The response to this derivative is quite distinctive although in general the response is much the same as with the methyl or ethyl guanidines. With most of the animals the pressure becomes subnormal after the primary rise and this effect may persist for a considerable period. The heart rate is affected to about the same extent as with ethanolguanidine and slightly greater than with methylguanidine, when using equivalent doses. The results of Experiment X XXVIII show the magnitude of the changes in the heart rate.

Experiment XXVIII. rabbit weighing 2.9 kg. Urethane anaesthesia.

Acetylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Max. Pressure	At 4 min.
5	292	246	296
After 2 mgm. per kg. atropine sulfate.			
5	316	280	304

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	At Min. Amplitude	At 1 min.	At 4 min.
5	68	96	104	80
After 2 mgm. per kg. atropine sulfate.				
5	78	140	132	100

The amplitude of the heart beat is slightly increased, much the same as with ethanolguanidine. Atropinization appears to have no effect upon the circulatory response to acetylguanidine, the blood pressure effect and the heart rate changes being the same as before.

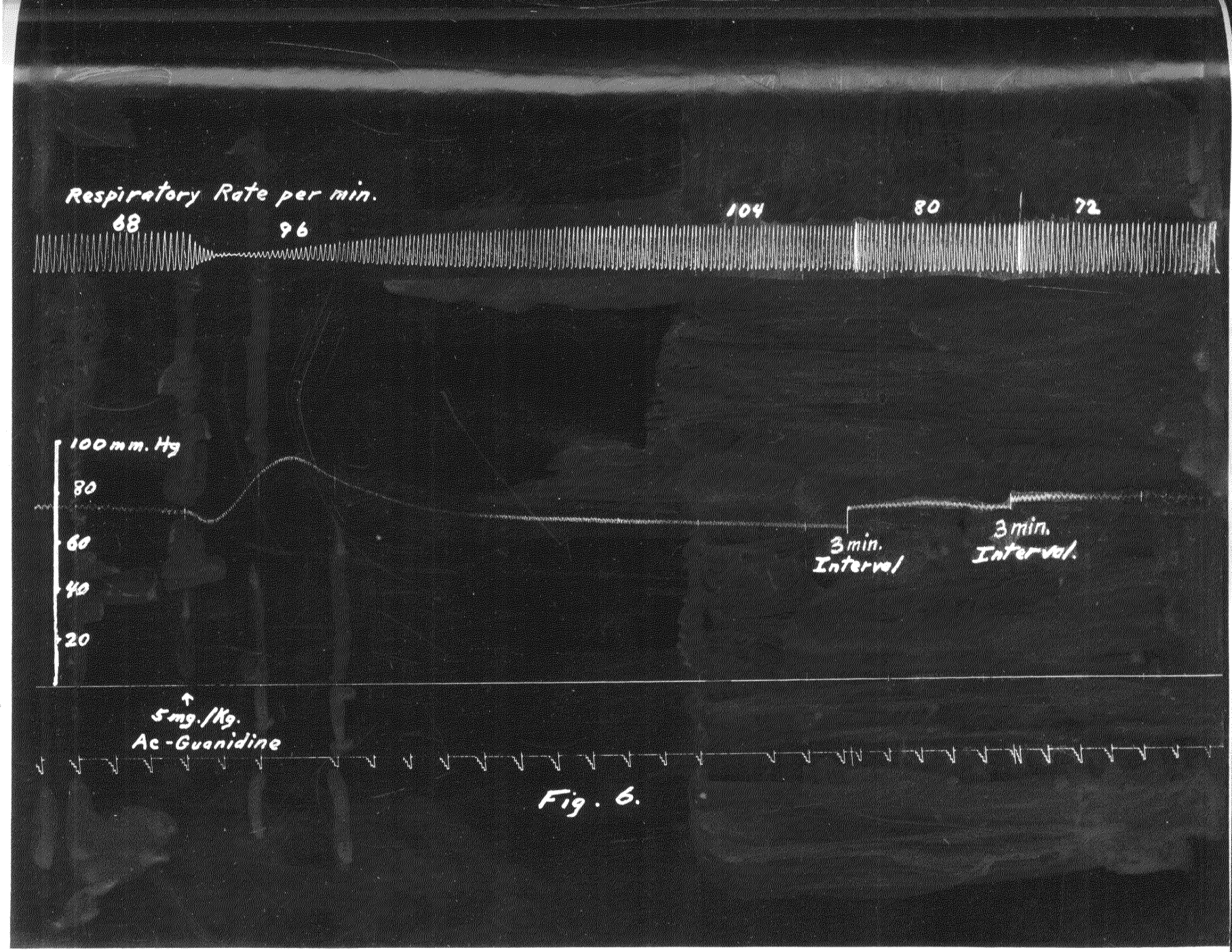
Respiratory Rate per min.
68
96
104
80
72
100 mm. Hg
80
60
40
20
3 min. Interval
3 min. Interval
5 mg./Kg. Ac-Guanidine

Fig. 6.

The respiration is markedly affected by acetylguanidine, the effect being greater than with methyl, ethyl, or ethanolguanidines. The decrease in amplitude immediately after injection is uniformly very marked with different animals, later the amplitude increases as the blood pressure is lowered. Experiment XXVIII shows the magnitude of the effect upon the respiratory rate, Atropinization, as with the other guanidine derivatives, increases the effect of acetylguanidine upon the respiratory rate, without causing a change in response with regard to amplitude.

Aminoguanidine. Fig. 7. shows the response after injecting aminoguanidine into the rabbit. Smaller amounts, 2 and 5 mgm. per kg. of the bas caused almost equivalent effects with other animals, the response seemed independent of the dosage. Generally the rise in pressure is lacking, there being an initial fall in pressure followed by a return to normal. The depressor effect of aminoguanidine is more marked than that of guanidine itself but is very similar. There is a considerable decrease in heart rate. Guanidine itself does not produce any appreciable change in heart rate.

The amplitude of the heart beat was increased coincidently with the decrease in rate and decrease in blood pressure. Atropinization appears to have no effect upon the circulatory response, the changes in pressure, rate and amplitude being the same after atropine as before. The effects of aminoguanidine on one of the rabbits are tabulated.below. This animal appeared to be very responsive to aminoguanidine, perhaps in part due to the fact that it was under only a very light anaesthesia.

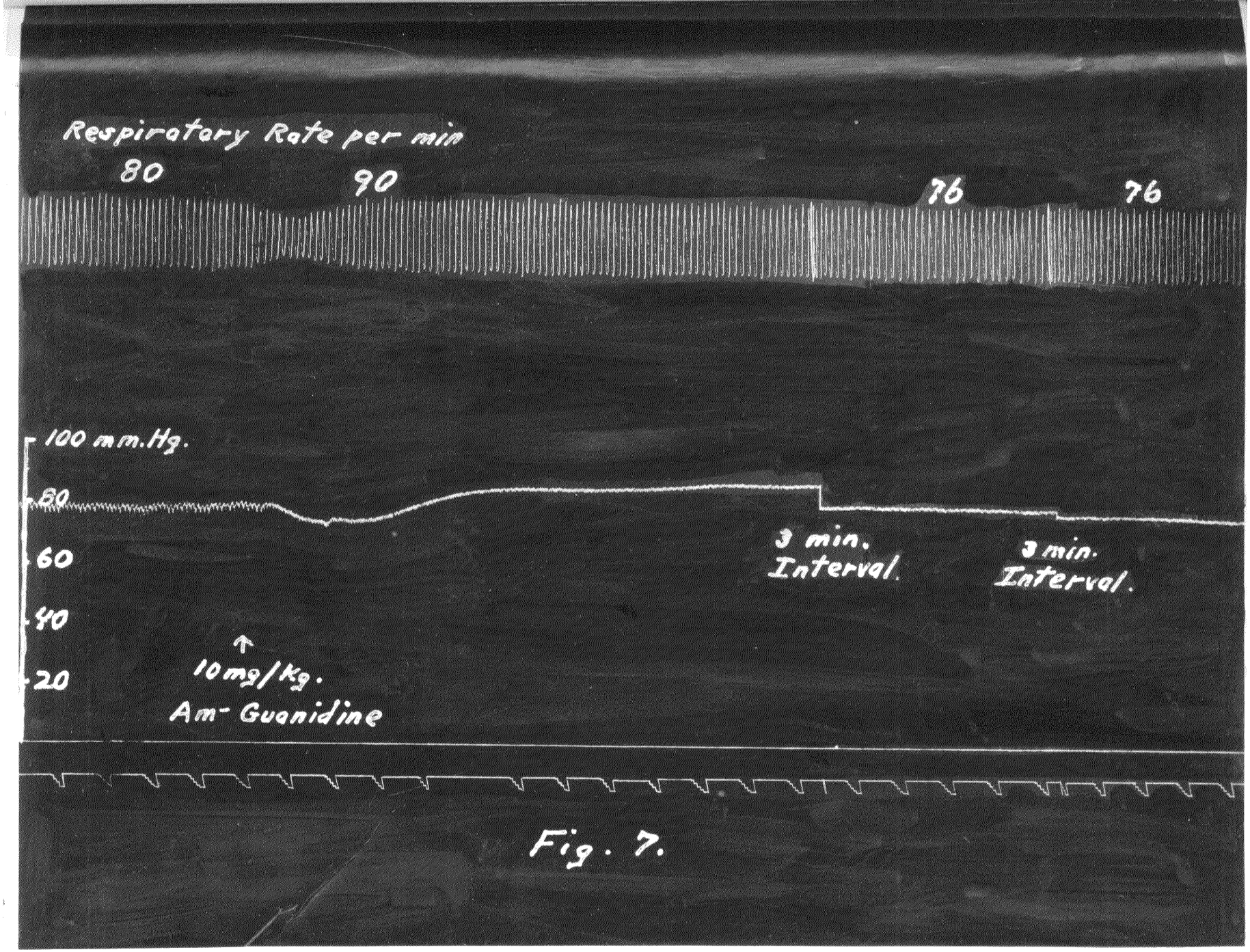
Respiratory Rate per min
80
90
76
76
100 mm.Hg.
80
60
40
20
3 min. Interval.
3 min. Interval.
10 mg/Kg.
Am-Guanidine

Fig. 7.

Experiment XXX. rabbit weighing 3.3 kg. Urethane anæsthesia.

Aminoguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Min. Pressure	At 1 min.	At 4 min.
2	328	276	300	318
After 2 mgm. per kg. atropine sulfate.				
2	300	270	282	300

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	At Min. Amplitude	At 1 min.	At 4 min.
2	180	224	222	204
After 2 mgm. per kg. atropine sulfate.				
2	150	200	180	144
10	132	240	252	204

The respiration is affected by aminoguanidine to a greater extent than with guanidine itself. The rate is much increased, the amplitude being decreased for a short period as with methyl and ethylguanidines. The change in amplitude and rate may be quite marked as it was in Experiment XXX., although in some cases, as in the experiment of Fig. 7., the changes may be more comparable to the changes produced by guanidine. Atropinization appears to have but little effect on the change in the respiration caused by aminoguanidine, although there does seem to be an increased effect after atropine in some cases.

Symmetric Diphenylguanidine. Fig.8. shows the response to diphenylguanidine in the rabbit. The circulatory response is in general similar to that of the other guanidines but more marked, 1 mgm. of the base having several times the effect of 20 mgm. of guanidine. The blood pressure response is characterized by a large

fall of pressure and the marked falls occuring at regular intervals during the minimal pressure, suggesting considerable cardiac depression. Only two animals have been used with this derivative but the response has been identical with both. The changes in heart rate are recorded in Experiment XXVII.

Experiment XXVII. rabbit weighing 2.9 kg. Urethane anaesthesia.

Symmetric Diphenylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	At Min. Pressure	At 1 min.	At 4 min.
1	336	292	292	320
After 2 mgm. per kg. atropine sulfate.				
1	318	180	294	312

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	After injection	At 1 min.	At 4 min.
1	124	136	124	96
After 2 mgm. per kg. atropine sulfate.				
1	96	102	98	102

The amplitude, between the large pulses, of the heart beat does not appear to be increased to any marked extent. Atropinization causes a considerable increase in the number of large pulses and consequently a more marked decrease in the heart rate. (See Experiment XXVII.

The amplitude of the respiration is only slightly affected by symmetric diphenylguanidine which is in contrast to the behavior of the other substituted guanidines. It may be that if large doses of diphenylguanidine could be used without the interference of toxic symptoms a more pronounced respiratory effect would be shown. Atropinization appears to decrease the effect of diphenylguanidine on the respiration.

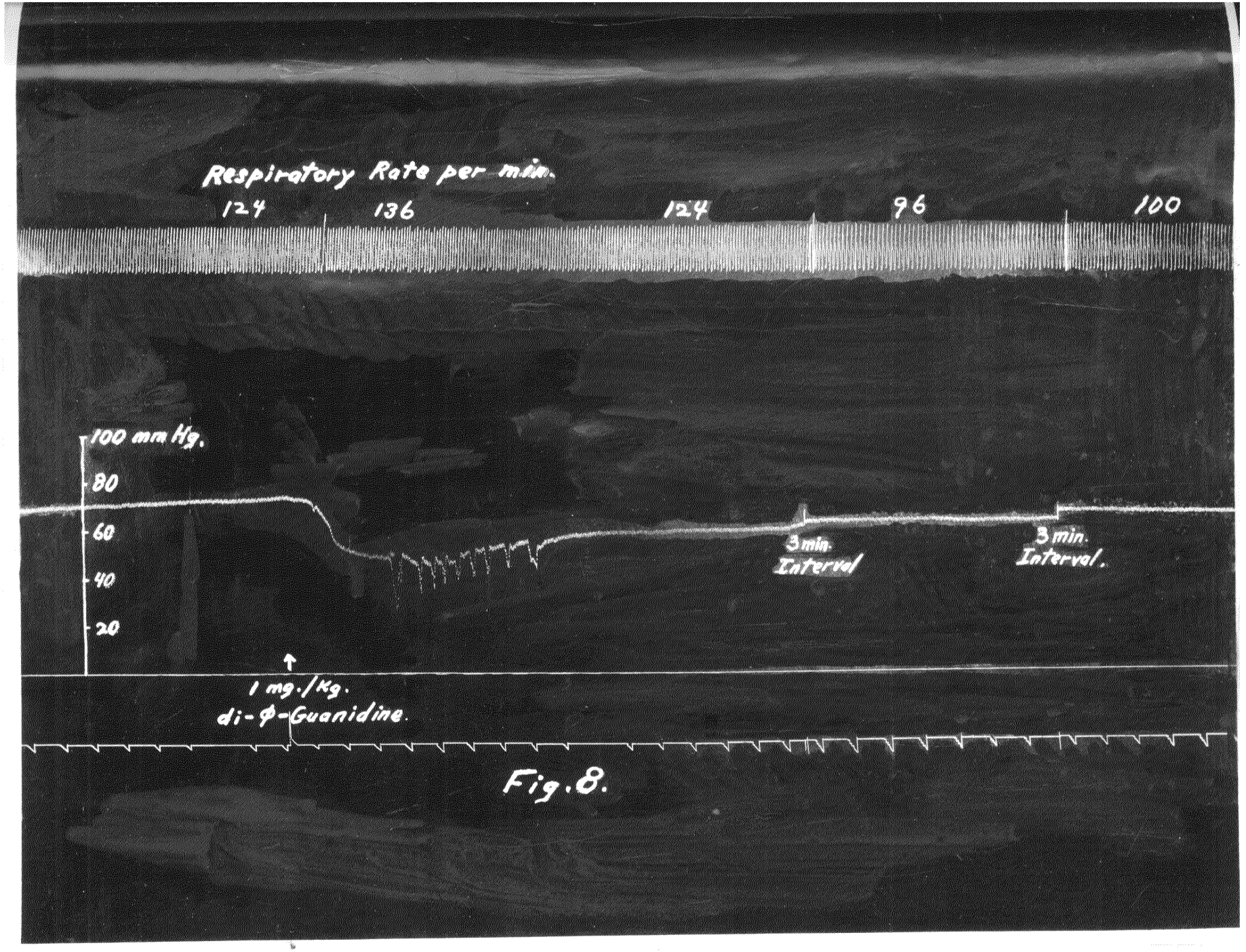
Respiratory Rate per min.
124
136
124
96
100
100 mm Hg.
80
60
40
20
3 min. Interval
3 min. Interval.
1 mg./kg.
di-ϕ-Guanidine.

Fig. 8.

Triphenylguanidine. Fig. 9. shows the response to triphenylguanidine in the rabbit. The circulatory response to this derivative is similar to that following the injection of diphenylguanidine although the effect is less marked. Larger doses are required to give equivalent blood pressure changes and the heart rate is changed to a lesser extent, even with the larger doses. The irregularity during the fall of blood pressure occuring with diphenylguanidine did not appear with the triphenyl derivative. The rise in pressure after the fall in pressure occuring immediately after injection is not very marked but is quite prolonged as is shown in Fig. 9. The tabulation of Experiment XXIX shows the magnitude of the effect of triphenylguanidine on the heart rate.

Experiment XXIX. rabbit weighing 2.9 kg. Urethane anaesthesia,

Triphenylguanidine Hydrochloride.

Circulatory effect.

Dose in mgm. of base per kg.	Heart Rate per minute. Normal	After injection	At 1 min.	At 4 min.
2	324	316	320	324
After 2 mgm. per kg. atropine sulfate.				
2	328	300	308	304

Respiratory effect.

Dose in mgm. of base per kg.	Respiratory Rate per minute. Normal	After injection	At 1 min.	At 4 min.
2	92	100	84	92
After 2 mgm. per kg. atropine sulfate.				
2	92	100	96	96

The amplitude of the heart beat is increased with the fall in pressure and returns to normal within a short time. Atropinization has little or no effect on the circulatory effect of triphenylguanidine. In the record of Experiment XXIX there appears to be an increased

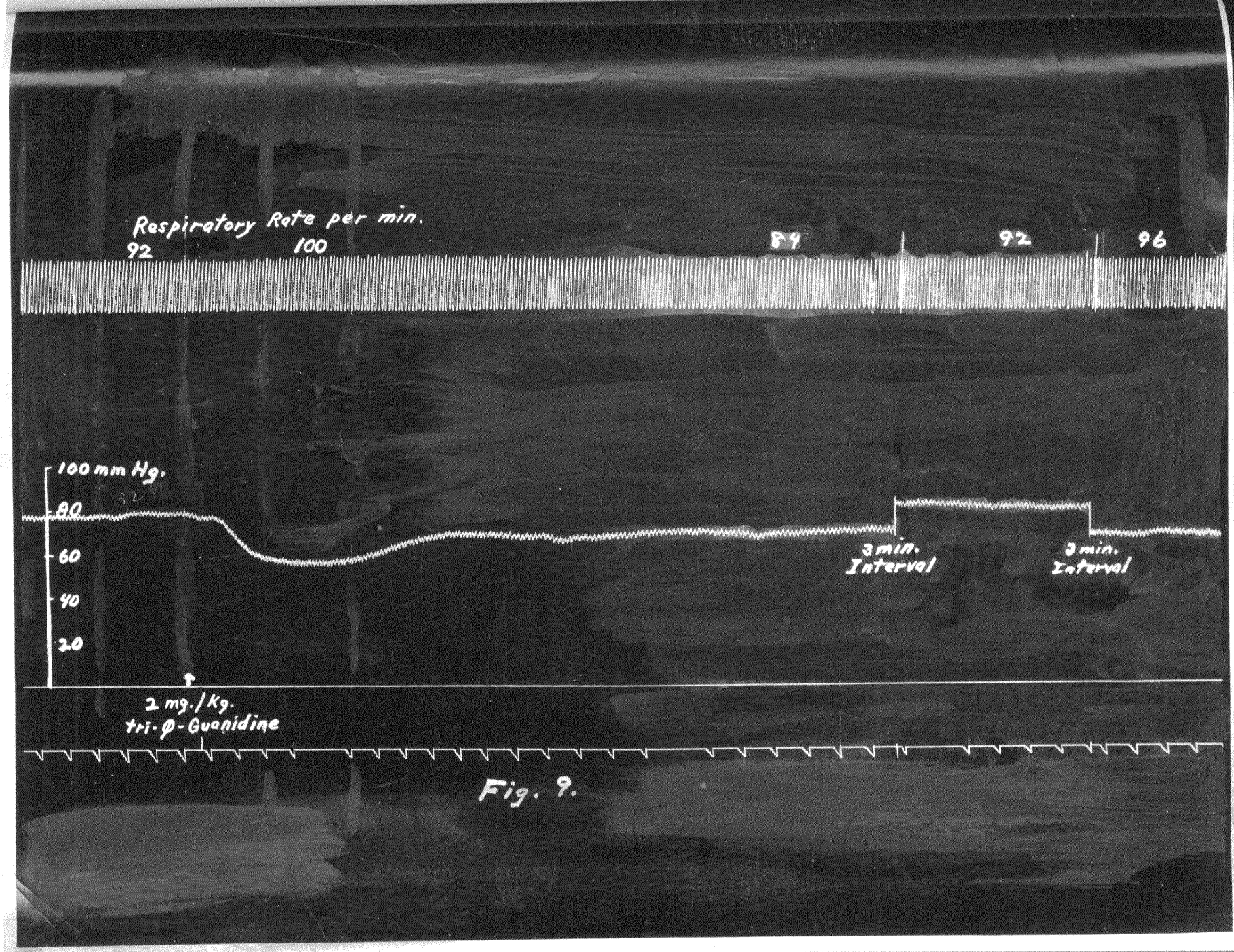
Respiratory Rate per min.
92
100
89
92
96
100 mm Hg.
80
60
40
20
3 min. Interval
3 min. Interval
2 mg./Kg.
tri-ϕ-Guanidine

Fig. 9.

change in the heart rate due to atropinization but in another experiment this effect did not appear.

The respiration is but slightly affected in rate and amplitude. A similar difficulty in observing a respiratory effect may be present here as with the diphenylguanidine, too small a dose to elicit respiratory effect may have been used.

Summary.

In general, the pressor response to guanidine, its alkyl, acetyl and amino derivatives consists of a fall in the blood pressure, followed by a rise that may be quite prolonged. The intensity of the effect of these derivatives is different, the acetyl, methyl, dimethyl, ethyl and ethanol guanidines being considerably more active than aminoguanidine or guanidine. The heart rate and amplitude of the beat are affected quite considerably by some of these derivatives, the effect being most marked with the ethyl and dimethyl derivatives. Methyl, ethanol, acetyl and amino guanidines have a less marked effect on the heart rate and amplitude of the beat, while guanidine itself has no appreciable effect.

The aryl guanidines, symmetric diphenylguanidine and triphenylguanidine are active with small doses. They cause a fall in blood pressure with a decrease in heart rate. The heart rate is very markedly affected with the diphenyl derivative, while the triphenyl derivative is not as active in this respect.

The respiratory response to the different guanidine derivatives studied was most marked with acetylguanidine. Methyl, dimethyl,

ethyl, ethanol and amino guanidines all have considerable effect on both the respiratory rate and amplitude, while the aryl derivatives used and guanidine itself are not marked in their action. The respiratory response consists of a short period of decreased amplitude and a long period of increased respiratory rate.

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III. Toxicity of some Guanidine Derivatives.

The toxicity of guanidine and of methylguanidine is of great interest to those who attempt to explain the toxic symptoms of protein shock as the poison action of a toxic material formed by the decomposition of protein. [1,2] It has further interest to those who look for an explanation of the tetany of parathyroidectomized animals in an alteration of the normal guanidine metabolism. [3]

The guanidine grouping is one of the few grouping known to occur in the protein molecule that may possess toxic properties and may therefore have wider interest in pathological considerations than has heretofore been given to it. A study has therefore been made of several guanidine derivatives to determine what effect certain substituents may have on the toxic action of the guanidine grouping. The study has been confined to a single experimental animal, the rat, and while it is very desirable that toxicity studies be made with different types of animals and different routes of injection it was preferred to study a larger number of derivatives, more exactly, using an arbitrary technic. The toxicity of guanidine, methylguanidine, and dimethylguanidine has been roughly covered for different animals by Frank, Stern and Nothmann [4] and by Klinger[5].

Experimental.

Rats weighing 100-150 g. were kept without food for from 16 to 24 hours previous to testing to establish an approximate metabolic level. The sexes were kept separate to avoid difficulty with pregnant females, but no difference in response was noted between the sexes. All injections were made subcutaneously into the lower

abdominal region, avoiding puncture of the peritoneum. The solutions used were made up to contain 5 mgm. of free base per 0.1 cc. (5-8% solutions), except in the case of the triphenylguanidine solution which contained but 2 mgm. of free base per 0.1 cc. The rats were kept for the 24 hours following injection in a warmed room and observation was carried out for this period only. A lethal dose was the dose causing death within 24 hours.

The guanidine derivatives used were all prepared as pure as possible in the form of hydrochlorides of the bases and recrystallized from alcohol. These hydrochlorides were analysed for chloride content and the content found agreed with that calculated for these compounds thus establishing their identity and purity. These compounds were further identified as picrates.

The results of the toxicity determinations are reported in the following table. The term " minimum lethal dose" designates the lowest dosage of a series of doses causing death within 24 hours, expressed as mgm. of free guanidine base per 100 g. animal body weight.

Derivative.	Minimum lethal dose
Guanidine	25
Methylguanidine	25
Asymmetric Dimethylguanidine	20
Ethylguanidine	20
Ethanolguanidine	200
Acetylguanidine	50
Aminoguanidine	200
Symmetric Diphenylguanidine	5
Triphenylguanidine	30

Discussion.

The table shows that guanidine, methylguanidine and asymmetric dimethylguanidine have much the same toxicity. Ethylguanidine has the same toxicity as the dimethyl derivative though one might expect a considerable difference. The increase in toxicity with the diphenyl derivative fits in with the usual greater toxicity of aromatic derivatives. However it is difficult to understand the fact that triphenylguanidine exhibits a toxicity slightly less than that of guanidine itself. The triphenyl derivative is less soluble and this may afford an explanation.

The fact that aminoguanidine is so slightly toxic is also difficult to understand, more especially when one considers aminoguanidine as a derivative of hydrazine, which is highly toxic. The solubility of aminoguanidine is high but it is of interest to note the slight solubility of the bicarbonate in this connection.

The lower toxicity of the oxygenated guanidine derivatives is also very considerable. Acetylguanidine appears to follow the general rule of lowered toxicity with acetylamines. It seems probable that ethanolguanidine is readily oxidized to the non-toxic creatine thus affording an explanation of its remarkably low toxicity.

The general guanidine effect is paralysis, occasional convulsions, dyspnoea and prostration. With the triphenyl derivative, even with one-tenth of the lethal dose, violent convulsive tremors occur, passing over the entire body. This effect is evidenced within an half hour and usually disappears within four hours. This effect of the triphenyl is not exhibited by the other derivatives and seems worthy of special

attention. The same very violent convulsions are exhibited in rabbits, using doses that are considerably sublethal. (5 to 20 mgm. of base per kg.)

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Toxicity Determinations.

Guanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	115	15	O.K. 12 and24 hours.
2	85	20	" " " " "
3	110	25	Dead 2½ hours.
4	175	30	O.K. 12 and 24 hours.
5	150	35	" " " " "
1	152	20	O.K. 12 and 24 hours.
2	105	25	" " " " "
3	110	30	" " " " "
4	90	35	" " " " "
5	100	40	Dead 2¼ hours.
1	90	20	O.K. 12 and 24 hours.
2	210	30	Dead 12 hours
3	130	40	V.sick 24 hours, chloroform
4	180	50	Dead 1½ hours.
5	150	60	Dead 1½ hours.

Methylguanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
2	110	15	O.K. 12 and 24 hours.
3	110	20	" " " " "
4	155	25	" " " " "
5	155	30	" " " " "
1	115	20	O.K. 12 and 24 hours.
2	145	25	Dead 12 hours.
3	70	30	Dead 2 hours.
4	115	35	O.K. 12 and 24 hours.
5	98	40	O.K. 12 and 24 hours.
1	170	20	O.K. 12 and 24 hours.
2	180	30	Dead 1½ hours.
3	180	40	Dead 2 hours.
4	140	50	V.sick 24 hours.chloroform
5	90	60	Dead 18 hours.

Toxicity Determinations.

Asymmetric Dimethylguanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	135	10	O.K. 12 and 24 hours.
2	85	15	" " " " "
3	90	20	Dead 2 hours.
4	105	25	O.K. 12 and 24 hours.
5	130	30	" " " " "
1	147	20	Dead 16 hours.
2	150	30	Dead 16 hours.
3	237	40	Dead 16 hours.
4	105	50	Dead 1½ hours.
5	83	60	Dead 1½ hours.

Ethylguanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	115	10	O.K. 12 and 24 hours.
2	75	15	" " " " "
3	100	20	" " " " "
4	115	25	Dead 2 hours.
5	150	30	Dead 1½ hours.
2	105	15	Dead 1½ hours
3	80	20	Dead 1 hour.
4	110	25	Conv. 1 hr. Dead 1½ hours.
5	90	30	Dead 40 minutes.
1	140	10	O.K. 12 and 24 hours.
2	91	15	" " " " "
3	95	20	" " " " "
1	97	20	Dead 2 hours.
2	106	25	Dead 2 hours.
3	105	30	Conv. 2 hrs. Dead 3 hours.

Toxicity Determinations.

Ethanolguanidine Hydrochloride,

No.	Weight in g.	mgm. base per 100 g.	Effect.
2	90	15	O.K. 12 and 24 hours.
3	90	20	" " " " "
4	115	25	" " " " "
5	135	30	" " " " "
2	100	30	O.K. 12 and 24 hours.
3	105	40	" " " " "
4	130	50	" " " " "
5	155	60	" " " " "
1	172	60	O.K. 12 and 24 hours.
2	140	80	" " " " "
3	140	100	" " " " "
4	95	120	" " " " "
1	83	150	O.K. 12 and 24 hours.
2	163	200	Dead 24 hours.

Acetylguanidine.Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	130	30	O.K. 12 and 24 hours.
2	135	40	" " " " "
3	150	50	" " " " "
4	90	60	Dead 24 hours.
5	120	70	O.K. 12 and 24 hours.
1	128	50	O.K. 12 hrs. Dead 24 hours.
2	110	60	Dead 4 hours.
3	120	70	O.K. 12 and 24 hours.
4	92	80	Dead 4 hours.
5	132	90	Dead 30 minutes
6	83	100	Dead 4 hours.

Toxicity Determinations.

Aminoguanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	72	20	Sick at start. Dead 1½ hrs.
2	145	40	O.K. 12 and 24 hours.
3	167	60	" " " " "
4	170	80	" " " " "
5	160	100	" " " " "
6	148	150	" " " " "
7	140	200	Dead 4 hours.

Symmetric Diphenylguanidine Hydrochloride,

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	112	1	O.K. 12 and 24 hours.
2	95	2	" " " " "
3	110	3	" " " " "
4	77	4	Paralysis ½-4 hrs. O.K. 24 hrs.
5	135	5	Paralysis ½-4 hrs. Dead 24 hrs.
1	82	2	O.K. 12 and 24 hours.
2	150	4	Paralysis ½ hr. O.K. 24 hrs.
3	120	8	Paralysis ½ hr. Dead 18 hrs.
4	150	12	Paralysis ½ hr. Dead 6 hours.

Triphenylguanidine Hydrochloride.

No.	Weight in g.	mgm. base per 100 g.	Effect.
1	100	2	Tremors ½-4 hrs. O.K. 24 hours.
2	95	4	" " " " " "
3	130	8	" " " " " "
4	145	12	" " " " " "
5	190	16	" " " " " "
1	139	20	Vio. Spasm ½-1 hr. O.K. 24 hours.
2	121	30	" " " " Dead 1½ hours.
3	119	40	" " " " Dead 45 min.
4	97	50	" " " " Dead 4 hours.

IV. Glucemic Effect of some Guanidine Derivatives.

Several years ago, Watanabe[1] made a very thorough study of blood changes occuring after the injection of guanidine into normal rabbits. He found among other things, that there was a decrease in the blood sugar content when nearly lethal doses were used. More recently Collip[2] in connection with his work on hypoglucemic substances in plants, injected guanidine into two animals and found a remarkably low blood sugar content at death. A plausible explanation of the hypoglucemic activity of guanidine is suggested by the fact that guanidine combines with glucose and other hexoses to form compounds which exhibit mutarotation in aqueous solutions.[3] Witzeman [4] following this idea studied the oxidation of glucose with hydrogen peroxide in the presence of guanidine but was unable to detect any specific effect due to guanidine. The work reported here would tend to show further that the hypoglucemic effect in the animal is a secondary effect, due perhaps to increased reflex excitability or convulsions.

Because of the unusual relationships between guanidine and the blood sugar of a normal animal it has occured to several investigators that insulin and guanidine may be related in some manner. Guanidine and several of its derivatives occur as physiological constituents of the body fluids and tissues and it seemed possible that there be some relationship between guanidine and the hormone. Recently Dubin and Corbitt [5] have observed effects of guanidine salts and methylguanidine sulfate on the blood sugar of normal rabbits and concluded that it is unlikely

that there is any relationship between these substances and insulin. At the time of learning of the work of Dubin and Corbitt the outhor was engaged in work somewhat more comprehensive in scope to determine whether guanidine derivatives might not exert a considerably greater hypoglucemic effect than that exerted by guanidine itself. Should a derivative be found having any considerable hypoglucemic activity, without a corresponding increase in activity, it would indirectly indicate that there may be a chemical relationship between insulin and the guanidine grouping. With this in mind several different guanidine derivatives were prepared that might be expected to exhibit a different degree of physiological activity than is exhibited by the parent guanidine. The effect of these different derivatives upon the blood sugar content of normal rabbits was determined.

Experimental.

The guanidine derivatives used were all prepared as pure as possible in the form of hydrochlorides of the bases and then recrystallized from alcohol. These hydrochlorides were analysed for chloride content and the content found agreed with that calculated for these compounds thus establishing their identity and purity. These compounds were further identified as picrates.

The testing of these substances was carried out on rabbits exclusively, using procedure that has been used in the testing of insulin preparations in this laboratory. The rabbits used were about 2 kg. in weight and were kept without food 20 hours previous

to the testing to establish an approximate metabolic level. All solutions were injected subcutaneously, using such concentrations of the hydrochlorides that the solutions contained 5 mgm. of the free base per 0.1 cc solution, with the exception of the triphenylguanidine solution which contained but 2 mgm. base per o.1 cc. Blood samples (0.1 cc) were taken 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours after the time of injection and analyzed, using the Hagendorn-Jensen technic.[6]

Since the chief object of this work was to determine if any of the guanidine derivatives had any considerable hypoglucemic activity, that is greater than guanidine itself, sublethal doses were used throughout. The effect of guanidine is only markedly apparent with nearly lethal doses and is not marked with any of the doses employed. However if any important hypoglucemic activity were exhibited by these derivatives, it should be apparent with the doses used.

Guanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	2.0	104	108	98	100	96	106	104
20	1.9	116	114	108	112	104	100	98
50	2.0	96	100	90	88	100	116	110
100	2.4	118	125	106	108	99	103	103

Methylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar inmmg. per 100 cc. blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	2.3	104	102	98	110	118	118	100
20	2.0	92	96	108	104	108	116	102
50	2.5	118	100	104	108	104	100	100

Asymmetric Dimethylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	2.3	108	126	112	100	112	102	99
20	c1.7	104	118	112	91	104	102	102
50	1.6	97	100	112	104	102	97	104

Ethylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	2.0	93	99	111	119	107	105	103
20	1.9	97	143	105	117	109	---	107
50	2.0	99	97	86	140	136	112	95

Ethanolguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	1.7	112	105	103	116	113	---	101
20	2.2	103	132	103	124	111	110	116
50	2.2	101	111	101	114	116	105	99
100	2.0	108	104	99	93	104	96	87
200	2.6	120	103	109	96	106	104	87

Acetylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	2.1	122	117	111	111	98	94	87
20	2.0	103	91	98	111	98	94	91
50	2.2	115	118	108	101	103	103	111

Aminoguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	1.7	106	108	112	93	97	95	102
20	1.5	112	132	124	83	108	112	112
50	1.8	106	112	100	104	104	100	116

Symmetric Diphenylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
10	1.9	117	122	109	109	115	105	107
20	2.1	107	129#	181	124	115	122	115
50	2.1	115	144$	155	124	120	104	115

Convulsions, dyspnoea, rapid breathing 1 hr. after injection.
$ Convulsions, dyspnoea, rapid breathing, prostration after 20 minutes.

Triphenylguanidine Hydrochloride.

mg. base per kg.	Wt. in kg.	Blood Sugar in mg. per 100 cc blood. Initial	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.
5	2.5	118	168#	168	113	126	124	111
10	2.2	141	118$	115	120	118	116	111
20	1.9	104	113&	113	109	107	107	109

Convulsions, intense tremors extending over entire body, beginning 20 minutes after injection and lasting 1 hour.
$ Convulsions, loss of coordination, etc 40 min. after injection.
& Same as other animals, convulsions, etc 40 min. after injection.

Discussion.

Several guanidine derivatives were tested to determine whether they would cause an appreciable glucemic effect. No definite changes in bllod sugar were observed and it may be concluded that they are without any considerable glucemic activity. If the glucemic effect of guanidine were a direct action in the animal body we should expect that a change in chemical constitution producing a change in physiological effect such as toxicity might result in a change of glucemic activity. It therefore seems probable that the hypoglucemic action of guanidine is a secondary manifestation, due perhaps to convulsions or increased reflex excitability.

The idea that insulin and guanidine may be chemically related seems very improbable in view of these studies, confirming previously reported work.

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OXIDATION OF GLUCOSE BY IODINE IN THE PRESENCE OF INSULIN.

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1. Purpose of this Investigation.

This investigation was undertaken with the purpose of determining whether insulin, alone or in the presence of certain animal fluids, has any influence upon glucose *in vitro*. The establishment of such an influence might have much significance in relation both to the study of carbohydrate metabolism and to the development of methods of assaying insulin.

For this purpose it seemed desirable to use a property for determining the glucose content which is highly specific for this substance and would not be likely to be shown in at all the same degree by products that might result from a change occurring under the influence of the insulin. The rate of oxidation of glucose ($C_6H_{12}O_6$) to gluconic acid ($C_6H_{12}O_7$) by iodine in nearly neutral solution seemed to be an especially suitable property; preliminary experiments on the oxidation of glucose, mannose, fructose, and sucrose by iodine having confirmed the results of previous investigators that the rates are widely different for these different compounds. A series of experiments was therefore made on the comparative rates of this oxidation, using in one case a pure glucose solution, and in other cases glucose solutions which had been previously treated with aqueous insulin extract, alone or mixed with liver extract, blood serum, or oxalated blood.

This investigation forms part of a series of researches on the chemical nature and behavior of insulin undertaken in this laboratory under the general direction of Prof. A. A. Noyes, to whom we desire to express our thanks for advice as to the work and for his aid in the preparation of it for publication. This investigation was

assisted on the financial side from the funds which have been made available by Dr. Bertnard Smith for the general prosecution of insulin researches in this laboratory. We are also indebted to Dr. Smith, and to his associate Dr. Howard West, for cordial cooperation and assistance on the biological sides of this investigation; also to Mr. Albert L. Raymond of this laboratory for many valuable suggestions.

2. Previous Researches.

Various investigators have already studied the reaction between sugars and iodine, with reference to the development of methods of analysis. Thus, Romijn[1] early showed that glucose is quantitatively oxidized by iodine in alkaline solution to gluconic acid. He found that aldoses, in general, are oxidized under these conditions, while ketoses remain unchanged; and he devised a method of sugar analysis based on this principle. As the results obtained with potassium or sodium hydroxide were rather irregular, he substituted sodium borate for the alkali. The reaction, although slow, proved to be quantitative in this weakly alkaline solution. Bougault[2] substituted sodium carbonate for the borate; and this gave a much more rapid oxidation and led to a satisfactory method of analysis. Willstätter and Schudel[3] found that the reaction proceeded smoothly to completion when 0.1 N solutions of iodine and sodium hydroxide in the proportion of 2:3 were used. Colin and Liévin[4] modified Bougault's procedure by using disodium hydrogen phosphate in the place of the carbonate. Cajori[5] has recently published an article in which he gives a method of separately determining glucose, fructose, sucrose, and maltose, in mixtures; this method being based on the different behaviors of these sugars towards iodine and towards cupric hydroxide.

The effect of insulin on glucose has been studied by Winter and Smith.[6] These investigators made a series of experiments

[1] Romijn, G., *Z. anal. Chem.*, 1897, xxxvi, 349.

[2] Bougault, J., *J. pharm. et chim.*, 1917, xvi, series 7, 97; *Compt. rend. Acad.*, 1917, clxiv, 1008.

[3] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, 1918, li, 780.

[4] Colin, H., and Liévin, O., *Bull. Soc. chim.*, 1918, xxiii, series 4, 403.

[5] Cajori, F. A., *J. Biol. Chem.*, 1922, liv, 617.

[6] Winter, L. B., and Smith, W., *Brit. Med. J.*, 1923, i, 12; *cf. J. Physiol.*, 1922–23, lvii, 100.

upon the change in optical rotation produced in glucose and fructose solutions by the addition of small amounts of insulin and liver extracts. They thought they detected, in the case of both sugars, an appreciable change, which reached a maximum in 2 to 4 days and was greatly increased by the addition of phosphate solution. They suggested that the function of insulin might be the activation of the enzyme (presumably present in the liver) which is responsible for the transformation of ordinary α β-glucose into the γ form. The γ sugars are known to be very reactive chemically: in summarizing their properties, Hewitt[7] notes their great activity toward oxidizing agents and their marked instability in the presence of acid and alkali.

3. Experimental Procedure and Materials.

The oxidations were carried out in a nearly neutral solution with an excess of iodine, so as to insure a nearly complete conversion of glucose into gluconic acid in a convenient period of time. To give the proper acidity, solutions 0.3 molal in $NaHCO_3$ or 0.15 molal in Na_2HPO_4 were used. In these solutions the reaction is practically complete at room temperature in $2\frac{1}{2}$ hours. About five times the theoretical amount of iodine was employed. The oxidations were carried on simultaneously in 100 cc. rubber stoppered conical flasks. Since the runs compared were always parallel, it was not thought necessary to maintain a fixed temperature. The temperature, however, did not vary over 2°C. throughout the entire work; and not more than a few tenths of a degree during a given set of oxidations.

The samples of sugars used were obtained as follows: sucrose from the U. S. Bureau of Standards; fructose, from Merck; and glucose and mannose, from Kahlbaum. Fresh solutions of the glucose were made up from time to time.

4. Rates of Oxidation of Glucose, Mannose, Fructose, and Sucrose.

The chemical equation expressing the oxidation of glucose or mannose by iodine is as follows:

$$C_6H_{12}O_6 + I_2 + H_2O = C_6H_{12}O_7 + 2HI$$

[7] Hewitt, J. A., *Brit. Med. J.*, 1923, i, 590.

The reaction rate at any moment should therefore be proportional to the prevailing concentrations both of the sugar and of the iodine, provided the temperature remains constant, and the hydrogen ion concentration, which has a large effect on the rate, also remains unchanged. That is, representing by A and B the initial concentrations of the sugar and iodine, respectively, by x the fraction of the sugar transformed at any time t, by $\frac{dx}{dt}$ the rate at which this fraction increases with the time, and by k the specific reaction rate at the given temperature and hydrogen ion concentration, the rate should be expressed by the equation:

$$A\frac{dx}{dt} = k\,(A - Ax)\,(B - Ax); \text{ or } \frac{dx}{dt} = k\,(1 - x)\left(\frac{B}{A} - x\right)$$

This equation yields on integration the following expression for the specific reaction rate.

$$k = \frac{0.4343}{t\,(B - A)} \log_{10} \frac{\left(\frac{B}{A}\right) - x}{\left(\frac{B}{A}\right)(1 - x)}$$

Reaction mixtures were made up in the case of each of these sugars by mixing the following solutions: 10.0 cc. of 0.1 per cent sugar solution; 10.0 cc. of 0.025 molal I_2 in 0.06 molal KI; 15.0 cc. of 1 molal $NaHCO_3$ or of 0.5 molal Na_2HPO_4; and 15.0 cc. of water. The mixtures were, therefore, initially 0.00111 molal in monosaccharide, 0.00500 molal in I_2, and either 0.3 molal in $NaHCO_3$ or 0.15 molal in Na_2HPO_4. Seven such mixtures of each sugar were placed in 100 cc. rubber stoppered conical flasks, and kept at the room temperature of 22–23°. From time to time one of the mixtures was removed, and 5 cc. of 6 N H_2SO_4 were added, whereby the reaction was stopped; the decrease in the free iodine content was then determined by titrating it with a standard $Na_2S_2O_3$ solution, which was 0.0200 molal in the $NaHCO_3$ experiments and 0.0189 molal in the Na_2HPO_4 experiments.

The results with glucose and mannose are given in Table I. In the columns headed 100 x are given the percentage of glucose oxidized as calculated from the decrease in iodine content; and in those headed k, the values of the specific reaction rate calculated by the above equation. The values given for the oxidation in the

$NaHCO_3$ solutions are the average of the three similar runs; those in the Na_2HPO_4 solutions represent one set of determinations.

It will be seen from Table I that, as would be expected for a bimolecular reaction taking place under constant catalytic conditions and temperature, the values of the specific reaction rate k are fairly constant for both glucose and mannose in the presence of the hydrocarbonate. In the presence of the hydrophosphate, however, the values of k show a progressive decrease.

Similar runs with fructose and sucrose showed an iodine consumption at the end of 160 minutes equivalent to only 6 per cent oxidation of the sugar.

TABLE I.

Rate of Oxidation of Glucose and Mannose by Iodine at 22–23°.

Time.	Glucose with $NaHCO_3$.		Glucose with Na_2HPO_4.		Mannose with $NaHCO_3$.		Mannose with Na_2HPO_4.	
	100 x	k	100 x	k	100 x	k	100 x	k
min.								
5	29.5	14.6	44.8	19.2	12.3	5.28	19.4	8.8
10	50.0	14.7	56.1	17.7	22.5	5.24	27.9	6.7
15	65.0	15.7	63.9	14.5	31.5	5.23	31.2	5.1
20	74.2	15.0	70.6	13.7	38.5	5.13	38.9	5.1
40	92.1	13.1	84.6	10.7	59.0	4.85	52.1	3.9
80	98.7		93.5		77.8	4.23	68.0	3.1
160	100.5		100.4		92.5		81.7	

These results conform with those of earlier investigators in that the aldoses are oxidized, while the ketose and biose remain practically unchanged. The small effect which is noted in the fructose and sucrose oxidations may well be due to experimental error. It is evident that even mannose and glucose differ greatly from each other in their rates of oxidation in both the hydrocarbonate and hydrophosphate solutions. The difference between the rates of glucose, mannose, and fructose should therefore be marked enough to indicate any transformation of glucose into isomeric hexoses which might occur in the presence of insulin.

5. Oxidation of Glucose in the Presence of Insulin.

The insulin used throughout the work was prepared by the process described by Collip[8] and was further purified by precipitation

[8] Collip, J. B., *Tr. Roy. Soc. Canada*, 1922, xvi, series 3, section v.

from aqueous solution by changing the hydrogen ion concentration. The precipitate was washed with ether and dried over calcium chloride. A 0.1 per cent solution of the dried substance was made up by dissolving it in approximately 0.001 N hydrochloric acid. Previous tests showed that the insulin was stable in this solution. The activity of this insulin was tested on rabbits several times: 0.3 cc. per kilo weight of rabbit lowered the blood sugar of a normal rabbit from 110 to 45 mg. per 100 cc. in 1 hour, and the convulsive dose was between 0.3 and 0.4 cc. The insulin was also found to retain most of its activity on standing for 1 hour in solutions with an alkalinity similar to that of the oxidation mixtures. In all the calculations, the iodine taken up by the insulin

TABLE II.

Oxidation of Glucose by Iodine in Presence of Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	25.3	23.4	+1.9	34.0	34.9	−0.9
10	43.7	40.7	+3.0	49.3	47.8	+1.5
15	52.6	53.8	−1.2	57.9	57.2	+0.7
20	61.5	60.7	−0.8	64.9	64.2	+0.7
40	80.6	80.2	+0.4	79.5	79.7	−0.2
80	95.7	96.3	−0.6	92.2	91.6	+0.6
160	101.9	101.2	+0.7	96.2	96.8	−0.6

introduced into the reaction mixture was subtracted from the total iodine consumed.

The method of procedure was exactly the same as that described above. The reaction mixtures were made up just as in the experiments on the oxidation of the sugars alone; except that there were always run side by side duplicate mixtures differing only in the respect that one contained in the 50 cc. volume 1 cc. of 0.1 per cent insulin solution, and the other contained no insulin. The temperature was 22-23°.

Table II contains the results of these experiments. The numbers in the first column show the time elapsed after the mixing of the solutions; those in the other columns denote the percentage of glucose transformed.

It is evident from these results that insulin alone has no appreciable effect upon the oxidation of glucose by transforming it into either a more or less chemically reactive form. This is true even though a considerable excess of insulin was present over that required for the oxidation of the 10 mg. of glucose; thus the quantity was sufficient to lower the blood sugar of a 3 kilo rabbit 50 mg. per 100 cc. of blood in 1 hour.

6. Oxidation of Glucose in the Presence of Liver Extract and Insulin.

Both alcoholic and aqueous liver extracts were used in these experiments. The method of procedure was the same as that followed in previous experiments with the exception that the reaction mixtures were allowed to stand for 1 hour with the liver extract and insulin, before the addition of the iodine. A temperature of 22-24° was maintained by immersing the solutions in a water bath. As before, one-half of the solutions contained 1 cc. of 0.1 per cent insulin solution (in place of 1 cc. of water), in addition to the other ingredients, which were as follows: 10.0 cc. of 0.1 per cent glucose solution, 15.0 cc. of a solution 1.0 molal in $NaHCO_3$ or 0.5 molal in Na_2HPO_4, 2.0 cc. of alcoholic or aqueous liver extract, 12.0 cc. of distilled water, and 10.0 cc. of a solution 0.05 N in I_2 and 0.06 N in KI.

The alcoholic liver extract was prepared in the following manner. Fresh beef liver was ground and intimately mixed with an equal volume of 95 per cent alcohol. The juice was pressed out of the mass, diluted to 4 volumes, and filtered. The filtered solution was used in the oxidations.

The aqueous liver extract was prepared as follows: Equal volumes of fresh beef liver and 0.9 per cent sodium chloride solution were ground together thoroughly with sand in a mortar. The juice was then pressed out, diluted to 4 volumes, and filtered.

Table III contains the results of the experiments in which the alcoholic extract was used; and Table IV those in which the aqueous extract was used. The figures in the first column show the length of time of the oxidations; those in the other columns show the cubic centimeters of 0.025 molal I_2 consumed in the various reaction mixtures. The second pair of experiments in each table was made with half the quantity of the liver extract.

These experiments show that glucose is unaffected by insulin in the presence of alcoholic or aqueous liver extract.

TABLE III.

Oxidation of Glucose by Iodine in Presence of Alcoholic Liver Extract and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	1.63	1.69	−0.06	1.50	1.50	0.00
10	2.37	2.38	−0.01	2.09	2.08	+0.01
15	2.86	2.83	+0.03	2.36	2.38	−0.02
20	3.48	3.44	+0.04	2.75	2.71	+0.04
40	4.23	4.14	+0.09	3.36	3.37	−0.01
80	5.34	5.29	+0.05	4.14	4.12	+0.02
160	6.23	6.17	+0.06	4.93	4.84	+0.09
5	1.75	1.81	−0.06	1.46	1.45	+0.01
10	2.46	2.45	+0.01	1.91	1.90	+0.01
15	3.01	2.97	+0.04	2.23	2.20	+0.03
20	3.26	3.23	+0.03	2.43	2.42	+0.01
40	4.34	4.33	+0.01	2.97	2.94	+0.03
80	5.21	5.21	0.00	3.71	3.69	+0.02
160	5.41	5.41	0.00	4.46	4.39	+0.07

TABLE IV.

Oxidation of Glucose by Iodine in Presence of Aqueous Liver Extract and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	3.96	3.93	+0.03	3.51	3.49	+0.02
10	4.98	4.98	0.00	4.10	4.09	+0.01
15	5.35			4.40	4.38	+0.02
20	5.67	5.66	+0.01	4.74	4.72	+0.02
40	6.44	6.43	+0.01	5.46	5.27	+0.19
80	7.07	7.09	−0.02	6.25	6.24	+0.01
160	7.39	7.45	−0.06	7.02	7.01	+0.01
5	2.26	2.25	+0.01	1.52	1.49	+0.03
10	2.71	2.72	−0.01	1.93	1.90	+0.03
15	3.10	3.10	0.00	2.26	2.23	+0.03
20	3.47	3.48	−0.01	2.55	2.53	+0.02
40	4.00	4.02	−0.02	2.89	2.88	+0.01
80	4.61	4.64	−0.03	3.24	3.24	0.00
160	4.88	4.92	−0.04	3.59	3.60	−0.01

7. Oxidation of Glucose in the Presence of Insulin and Blood.

In order to make the work more complete, the effect of insulin on the oxidation of glucose in the presence of the blood serum and oxalated blood was studied. As in the other experiments, the results consistently indicated no change in the glucose on the addition of insulin.

The method of procedure was exactly the same as that followed in the work with liver extract. The reaction mixtures were changed only by substituting blood or blood serum for the liver extract.

Table V contains the results of the set of experiments in which 0.2 cc. of oxalated blood was added to each of the reaction mixtures. The temperature was 23.2–23.4°

TABLE V.

Oxidation of Glucose in Presence of Oxalated Blood and Insulin.

Time.	Solution 0.30 molal in $NaHCO_3$.			Solution 0.15 molal in Na_2HPO_4.		
	Without insulin.	With insulin.	Difference.	Without insulin.	With insulin.	Difference.
min.						
5	2.92	2.91	+0.01	2.51	2.48	+0.03
10	3.48	3.48	0.00	2.94	2.92	+0.02
15	3.83	3.89	−0.06	3.24	3.22	+0.02
20	4.07	4.12	−0.05	3.44	3.42	+0.02
40	4.75	4.77	−0.02	4.01	4.00	+0.01
80	5.20	5.23	−0.03	4.60	4.57	+0.03
160	5.64	5.64	0.00	5.03	5.05	−0.02

SUMMARY.

It was first shown by this investigation that, in confirmation of the results of others, the rate of oxidation of various sugars by iodine in solutions of $NaHCO_3$ or Na_2HPO_4 varies greatly with the nature of the sugar; thus mannose was oxidized only about one-third as fast as glucose, and fructose and sucrose were scarcely oxidized at all, under the conditions of the experiments. This indicated that a study of the relative rates of oxidation of glucose before and after treatment of it with insulin would furnish a sensitive means of determining whether any of the glucose had been transformed by it into any other substance, even into a stereomeric hexose.

Strictly comparable experiments were therefore made with glucose alone, with mixtures of it with insulin, with insulin and liver extract, or with insulin and blood serum or oxalated blood. In no case was any difference detected in the rate at which the iodine is consumed. This shows that no appreciable reaction takes place between glucose and insulin even in the presence of the animal fluids mentioned. It indicates, therefore, that the metabolic process must be more complicated in character; also that there is little promise of developing a method of assay for insulin on the basis of its action on glucose in glass.

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COMPARATIVE METHODS OF PROTEIN EXTRACTION WITH CHEMICAL AND CLINICAL STUDIES *

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Protein skin tests are a generally accepted method of diagnosis in allergic diseases. It is known that they furnish reliable evidence in determining the etiologic factors concerned in allergic manifestations. This reliability is based on evidence that may be demonstrated both clinically and experimentally. On the clinical side, we know that typical skin or systemic reactions are more or less constant for each specific protein in a given sensitive individual, that removal of this protein relieves the allergic symptoms, and that by small, graduated doses it is possible to immunize a sensitive individual against this protein's injurious effects. On the laboratory and experimental side, we know that many proteins give a precipitin or a complement fixation test with the serum of the individual in whom they produce allergic symptoms; that it is possible to sensitize a hitherto nonsensitive individual to a given protein, and that there is an analogous and, in almost every way, similar train of evidence obtainable in experimental animals as demonstrated in the production of anaphylactic shock. This, then, summarizes the evidence on which is based the general acceptance of the protein skin tests.

Throughout the vast amount of scientific work that has been done to make possible the foregoing dogmatic statements, the assumption has arisen that proteins and only proteins are capable of producing anaphylactic or

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allergic reactions. Indissolubly bound up with this assumption is that of the specificity of immunologic phenomena. One cannot be discussed without the other. In general, they must be accepted, yet evidence is constantly being gathered attacking their fundamental basis. Thus, Manwaring [1] has been able to produce an anaphylactic reaction in a guinea-pig lung with Vaughan's split protein; Wells [2] speaks of the specificity of lipoid substances, and recently Landsteiner [3] has produced anaphylaxis by sensitizing an animal to one protein containing an azo group, and using as the anaphylactc dose another protein to which the same azo group had been attached. Moreover, peptone shock and nonspecific protein shock, questioning in the one instance the protein basis and in the other the protein specificity of immunologic phenomena, have long been known. Add to this our inability to produce a single protein of constant chemical composition, and we have sufficient evidence of the incompleteness of our knowledge of proteins.

We believe that a further study of the chemical nature of protein, checked up with clinical observations, will throw considerable light on what is as yet an obscure subject. In all the work on allergy, the first unknown factor is the protein, the basic tool with which we work. Not only is its nature unknown, but the methods of obtaining proteins in the great majority of cases are based, to a great extent, on purely empiric grounds. The substances obtained by various methods of extraction are called proteins: they produce skin reactions; they produce constitutional reactions, and, in the laboratory and on experimental animals, they pass the criticism of producing those reactions which we have learned to recognize as criteria of immunity or the lack of it. Numerous methods are used to extract these substances. Yet, so far as we know, no systematic study has been made with the greater number of the proteins used in protein skin tests to determine whether we are here dealing with one or many proteins, or whether, in fact, we are dealing with proteins at all.

In an attempt to attack this problem rationally, we have undertaken some elementary studies in the use of

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different solvents for the extraction of these substances. As stated, many methods have been described by Goodale,[4] Coca[5] and others, for dissolving out these substances from the animal danders, foods and pollens, with the specific idea of using them for protein skin tests. The production of dry proteins for this purpose, using water and physiologic sodium chlorid solution as extractives, has been described by Wodehouse,[6] Osborne,[7] Mendel and Harris have described methods for extracting proteins from cereals, and Wells and Osborne[8] for extracting proteins from vegetables. Methods for obtaining the various proteins of eggs and milk have long been known, the products so obtained have been found satisfactory in their application as antigens for the protein skin tests.

This paper is a preliminary report. We have not attempted to cover the general subject of extraction of all substances responsible for allergic skin reactions, but have studied the extraction of foodstuffs and animal epidermal structures. No consideration has been given to the extraction of pollens or of bacterial preparations. The task of securing suitably sensitive persons who are willing to be repeatedly tested, together with the difficulty of securing uniformly consistent skin reactions on a given individual, causes our problem to be complex, thus adding to the difficulty of obtaining sufficient evidence on the subject to be conclusive.

So far as the character of the substances causing the allergic skin reactions are known, they are an intimate part of the protein molecule. For this reason, solvents were used in this work that would be expected to extract differentially the several classes of protein that might be present. The main facts regarding the solubilities of the simple uncoagulated proteins are as follows:

1. Albumins, some globulins, histons and protamins are soluble in water.

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2. Globulins, together with most of the proteins soluble in water, dissolve in dilute (from 1 to 10 per cent.) sodium chlorid solution.

3. Prolamins are soluble in alcohol (from 70 to 90 per cent.).

4. Albuminoids are not soluble in dilute acid, dilute alkali, water, salt solutions or alcohol.

These are only qualitative statements of solubility behavior, and, although not exact in nature, probably afford the most satisfactory characterization of the different classes of protein.

With these statements in mind, the following solvents were chosen for the extraction of the substances giving the allergic skin reactions:

A. 1 per cent. sodium chlorid solution.
B. 5 per cent. sodium chlorid solution.
C. 10 per cent. sodium chlorid solution.
D. 0.5 per cent. sodium hydroxid solution.
E. 0.5 per cent. sodium hydroxid containing 5 per cent. sodium chlorid.
F. Coca's solution,[5] which is a dilute sodium bicarbonate, phenol (carbolic acid) solution.
G. 10 per cent. alcohol.
H. 70 per cent. alcohol.
I. 95 per cent. alcohol.

Solutions F and G, Coca's solution, and 10 per cent. alcohol solution serve as comparison solutions; Coca's solution being a satisfactory solvent for foodstuffs and animal emanations, and 12 to 14 per cent. alcohol being reported as a satisfactory solvent for animal allergens by Goodale,[4] Wodehouse[9] and others. The 95 per cent. alcohol solvent was used because it would not be expected to dissolve protein but should dissolve many protein split products, glucosids, nitrogen extractives and similar classes of compounds that might contribute to the allergic reaction.

One or more of each of the following food groups was extracted: cereals, fruits, meats, nuts, seafoods and vegetables. Horse dander and dog hair were used for the epidermal structure extracts. The proportion of material to 20 c.c. of solvent in making the extracts is stated in Table 1. The extracts were made by con-

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tinuous shaking with the solvent for one hour at room temperature, centrifugating out the major portion of undissolved material, and finally filtering through paper. The solutions were preserved under toluene in stoppered bottles, and kept in a refrigerator when not in use. Some precipitation occurred, on standing, in most of the extracts made, but all of these solutions appeared to have retained their activity over the period of about three months during which they were in use.

All the solutions were analyzed for their nitrogen contents by a colorimetric micro-Kjeldahl-Nessler method (Table 2). The designation nonprotein nitrogen has been arbitrarily applied to the nitrogen content

TABLE 1.—*Character and Proportion of Material in Grams Treated with Twenty Cubic Centimeters of Solvent*

Material	Weight in Grams
Cabbage, fresh, ground	10
Banana, ripe, fresh, ground	10
Rye, milled flour	1
Horse dander, ether extracted	1
Chicken, bones removed, meat ground	5
Peas, dried, ground	1
Dog hair, ether extracted	..
Peanuts, ground, ether extracted	1
Salmon, fresh, bones removed, ground	5
Walnut, ground, ether extracted	1
Wheat, whole, milled flour	1

present that is not precipitated in a 2 per cent. phosphotungstic acid solution (Table 3). This designation, though purely arbitrary, is as nearly the proper one as we can determine by a consideration of the literature on the precipitation behavior of protein and nonprotein nitrogenous compounds.

Consulting Table 2, we see that solutions D and E, viz., the 0.5 per cent. sodium hydroxid and the 0.5 per cent. sodium hydroxid plus 5 per cent. sodium chlorid, consistently gave the largest yield of total nitrogen per milligram per cubic centimeter. Comparing this with Table 3, which tabulates in the same manner for each substance and each extractive the difference between the total nitrogen and the nonprotein nitrogen per milligram per cubic centimeter, thus giving presumably the amount of protein nitrogen present, we find again that in almost every instance Solutions D and E were most efficient.

TABLE 2.—*Total Nitrogen in Milligrams per Cubic Centimeter*

Solvent	Cabbage	Banana	Rye	Pea	Horse Dander	Chicken	Dog Hair	Peanut	Salmon	Walnut	Wheat
A. 1% sodium chlorid	0.84	0.79	0.91	0.92	2.02	1.92	0.31	1.29	2.50	0.29	0.45
B. 5% sodium chlorid	0.78	0.78	0.85	1.14	2.01	2.61	0.15	3.00	2.55	0.43	0.43
C. 10% sodium chlorid	0.72	0.79	0.82	1.02	1.89	1.98	0.15	1.50	3.00	0.32	0.38
D. 0.5% sodium hydroxid	0.98	0.92	0.90	1.85	2.40	4.72	0.41	5.32	5.10	3.21	1.08
E. 0.5% sodium hydroxid + 5% sodium chlorid	0.96	0.88	0.90	1.43	2.19	3.79	0.38	4.30	3.50	2.35	1.00
F. Coca's solution	0.84	0.78	0.64	1.04	2.01	1.92	0.17	2.08	1.02	0.40	0.41
G. 10% alcohol	0.80	0.80	0.62	0.72	1.98	1.44	0.16	2.20	1.17	0.32	0.40
H. 70% alcohol	0.92	0.60	0.60	0.50	1.72	0.63	0.10	0.30	0.81	0.26	0.61
I. 95% alcohol	0.78	0.58	0.40	0.40	1.40	0.54	0.04	0.24	0.81	0.22	0.25

TABLE 3.—*Difference Between Total Nitrogen and Nonprotein Nitrogen in Milligrams per Cubic Centimeter*

Solvent	Cabbage	Banana	Rye	Pea	Horse Dander	Chicken	Dog Hair	Peanut	Salmon	Walnut	Wheat
A. 1% sodium chlorid	0.35	0.51	0.72	0.75	1.72	1.63	0.17	1.07	....	0.18	0.38
B. 5% sodium chlorid	0.29	0.58	0.66	0.97	1.69	2.32	0.01	2.74	....	0.32	0.36
C. 10% sodium chlorid	0.23	0.51	0.63	0.85	1.59	1.69	0.01	1.24	....	0.21	0.31
D. 0.5% sodium hydroxid	0.35	0.64	0.70	1.63	2.07	4.44	0.20	5.09	4.84	3.07	0.97
E. 0.5% sodium hydroxid + 5% sodium chlorid	0.29	0.62	0.69	1.21	1.89	3.52	0.20	4.06	3.22	2.22	0.91
F. Coca's solution	0.32	0.56	0.43	0.71	1.73	1.60	0.02	1.80	0.67	0.29	0.34
G. 10% alcohol	0.21	0.80	0.44	0.56	1.61	1.15	0.01	2.03	0.82	0.21	0.31
H. 70% alcohol	0.11	0.60	0.41	0.31	1.28	0.34	....	0.13	0.46	0.15	0.53
I. 95% alcohol	....	0.26	0.23	0.40	0.92	0.25	....	0.07	0.46	0.11	0.17

TABLE 4.—*Method of Tabulating Results of Skin Reactions with Horse Dander*

Solvent	Total Nitrogen, Mg. per C.c.	Nonprotein Nitrogen, Mg. per C.c.	Names of Subjects: M. J. M.	C. F.	Liv.	R. D.	W. D.	M. M.	P. R.
A. 1% sodium chlorid	2.02	....	+++	++++	+++	+++	++++	+	++++
B. 5% sodium chlorid	2.01	0.30	+++	++++	++++	+++	++++	++++	+++
C. 10% sodium chlorid	1.89	....	+++	++++	++++	++++	++++	++	++++
D. 0.5% sodium hydroxid	2.40	0.33	0	++	+++	++++	+++	++++	0
E. 0.5% sodium hydroxid + 5% sodium chlorid	2.19	0.30	0	+	+++	++++	+++	+	0
F. Coca's solution	2.01	0.28	+++	0	++++	++++	++++	+++	+++
G. 10% alcohol	1.98	0.37	+++	+++	++++	++++	++++	+++	++++
H. 20% alcohol	1.72	0.44	0	0	0	+++	+	++	0
I. 95% alcohol	1.40	0.48	0	++	0	++	+	0	0

The skin tests reported were made by a single observer by the usual cutaneous scratch method on the arm or back of the person tested. A complete set of solutions of one material was tested at one time, in order to minimize the variations in reactions of the individual as much as possible. The markings indicating the reactions have the following significances: Zero denotes no reaction; + a questionable reaction; + + a distinctly positive but weak reaction; + + + a good positive reaction, and + + + + a strongly positive reaction, usually more than 1.5 cm. in diameter. In addition, it might be stated that no skin manifestation was called a reaction unless it showed definitely the pseudopods that we have learned to associate with the typical allergic skin reaction (Table 4).

TABLE 5.—*Summary of Skin Reactions Obtained by Using Various Extracting Fluids*

	A	B	C	D	E	F	G	H	I
Cabbage.....	2/4	4/4	3/4	0/4	2/4	1/4	0/4	2/4	1/4
Banana.....	2/2	2/2	2/2	2/2	2/2	2/2	1/2	1/2	1/2
Rye.....	2/3	3/3	3/3	1/3	0/3	2/3	2/3	0/3	0/3
Pea.....	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1
Horse dander...	7/7	7/7	7/7	5/7	5/7	6/7	7/7	3/7	3/7
Chicken.....	1/1	1/1	1/1	1/1	1/1	0/1	0/1	1/1	1/1
Dog hair.....	2/4	3/4	3/4	1/4	3/4	1/4	3/4	1/4	1/4
Peanut.....	3/3	3/3	2/3	2/3	2/3	3/3	3/3	1/3	1/3
Salmon.....	1/2	1/2	1/2	0/2	2/2	1/2	1/2	0/2	0/2
Wheat.....	3/3	3/3	3/3	0/3	3/3	2/3	2/3	2/3	2/3
Walnut.....	3/4	3/4	3/4	1/4	2/4	3/4	1/4	0/4	0/4
Totals.....	27/34	31/34	29/34	14/34	22/34	21/34	20/34	11/34	10/34

Table 5 is a summary of the results obtained by using the solutions described above in the protein skin tests. In this table, the denominator of each fraction indicates the number of individual tests with each solution for each substance, and the numerator the number of positive skin reactions obtained. Thus, the total number of tests for each solution of each substance was thirty-four. It is seen on consulting this table that, from the clinical side, the most efficient extractives were Solutions A (1 per cent. sodium chlorid), B (5 per cent. sodium chlorid) and C (10 per cent. sodium chlorid), which gave the highest total number of positives, in spite of the fact that they do not contain either as much total nitrogen or protein nitrogen by difference as do Solutions D and E. This, of course, is probably explained by the fact that the alkali content in Solutions

D and E is sufficient to hydrolyze the proteins or other active substances present with consequent loss of activity. The least effective extractive proved to be the 95 per cent. alcohol, as would be expected. However, inspection of the table shows that although alcohol in this concentration is a very poor solvent for proteins, yet the considerable number of reactions obtained with many of the extracted materials indicates that some active substances, whether proteins or not, are extracted by this solvent.

SUMMARY AND CONCLUSIONS

1. Owing to our fragmentary knowledge of protein chemistry as applied to allergy, further research into the chemical nature of proteins and their clinical application in allergy is needed.

2. A systematic study of the actions of various chemical solvents in extracting substances capable of producing skin reactions indicates that the solvent known to be most effective in extracting the natural proteins without denaturization, namely, salt solution up to 10 per cent., is also most effective in obtaining skin reacting substances.

3. Neither the total nitrogen content nor the so-called protein nitrogen content by difference can be used to foretell the activity of a solution.

4. Ninety-five per cent. alcohol, although not a protein solvent, extracts substances that produce allergic skin reactions.

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In partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Chemistry.

CALIFORNIA INSTITUTE OF TECHNOLOGY
Pasadena, California.

1926.

COMPARATIVE METHODS OF PROTEIN EXTRACTION WITH CHEMICAL AND CLINICAL STUDIES *

GEORGE PINESS, M.D.
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AND
GORDON A. ALLES, B.S.
LOS ANGELES

Protein skin tests are a generally accepted method of diagnosis in allergic diseases. It is known that they furnish reliable evidence in determining the etiologic factors concerned in allergic manifestations. This reliability is based on evidence that may be demonstrated both clinically and experimentally. On the clinical side, we know that typical skin or systemic reactions are more or less constant for each specific protein in a given sensitive individual, that removal of this protein relieves the allergic symptoms, and that by small, graduated doses it is possible to immunize a sensitive individual against this protein's injurious effects. On the laboratory and experimental side, we know that many proteins give a precipitin or a complement fixation test with the serum of the individual in whom they produce allergic symptoms; that it is possible to sensitize a hitherto nonsensitive individual to a given protein, and that there is an analogous and, in almost every way, similar train of evidence obtainable in experimental animals as demonstrated in the production of anaphylactic shock. This, then, summarizes the evidence on which is based the general acceptance of the protein skin tests.

Throughout the vast amount of scientific work that has been done to make possible the foregoing dogmatic statements, the assumption has arisen that proteins and only proteins are capable of producing anaphylactic or

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allergic reactions. Indissolubly bound up with this assumption is that of the specificity of immunologic phenomena. One cannot be discussed without the other. In general, they must be accepted, yet evidence is constantly being gathered attacking their fundamental basis. Thus, Manwaring [1] has been able to produce an anaphylactic reaction in a guinea-pig lung with Vaughan's split protein; Wells [2] speaks of the specificity of lipoid substances, and recently Landsteiner [3] has produced anaphylaxis by sensitizing an animal to one protein containing an azo group, and using as the anaphylactc dose another protein to which the same azo group had been attached. Moreover, peptone shock and nonspecific protein shock, questioning in the one instance the protein basis and in the other the protein specificity of immunologic phenomena, have long been known. Add to this our inability to produce a single protein of constant chemical composition, and we have sufficient evidence of the incompleteness of our knowledge of proteins.

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This paper is a preliminary report. We have not attempted to cover the general subject of extraction of all substances responsible for allergic skin reactions, but have studied the extraction of foodstuffs and animal epidermal structures. No consideration has been given to the extraction of pollens or of bacterial preparations. The task of securing suitably sensitive persons who are willing to be repeatedly tested, together with the difficulty of securing uniformly consistent skin reactions on a given individual, causes our problem to be complex, thus adding to the difficulty of obtaining sufficient evidence on the subject to be conclusive.

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2. Globulins, together with most of the proteins soluble in water, dissolve in dilute (from 1 to 10 per cent.) sodium chlorid solution.

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F. Coca's solution,[5] which is a dilute sodium bicarbonate, phenol (carbolic acid) solution.
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Solutions F and G, Coca's solution, and 10 per cent. alcohol solution serve as comparison solutions; Coca's solution being a satisfactory solvent for foodstuffs and animal emanations, and 12 to 14 per cent. alcohol being reported as a satisfactory solvent for animal allergens by Goodale,[4] Wodehouse[9] and others. The 95 per cent. alcohol solvent was used because it would not be expected to dissolve protein but should dissolve many protein split products, glucosids, nitrogen extractives and similar classes of compounds that might contribute to the allergic reaction.

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Salmon, fresh, bones removed, ground	5
Walnut, ground, ether extracted	1
Wheat, whole, milled flour	1

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Consulting Table 2, we see that solutions D and E, viz., the 0.5 per cent. sodium hydroxid and the 0.5 per cent. sodium hydroxid plus 5 per cent. sodium chlorid, consistently gave the largest yield of total nitrogen per milligram per cubic centimeter. Comparing this with Table 3, which tabulates in the same manner for each substance and each extractive the difference between the total nitrogen and the nonprotein nitrogen per milligram per cubic centimeter, thus giving presumably the amount of protein nitrogen present, we find again that in almost every instance Solutions D and E were most efficient.

TABLE 2.—*Total Nitrogen in Milligrams per Cubic Centimeter*

	Solvent	Cabbage	Banana	Rye	Pea	Horse Dander	Chicken	Dog Hair	Peanut	Salmon	Walnut	Wheat
A.	1% sodium chlorid	0.84	0.79	0.91	0.92	2.02	1.92	0.31	1.29	2.50	0.29	0.45
B.	5% sodium chlorid	0.78	0.78	0.85	1.14	2.01	2.61	0.15	3.00	2.55	0.43	0.43
C.	10% sodium chlorid	0.72	0.79	0.82	1.02	1.89	1.98	0.15	1.50	3.00	0.32	0.38
D.	0.5% sodium hydroxid	0.98	0.92	0.90	1.85	2.40	4.72	0.41	5.32	5.10	3.21	1.08
E.	0.5% sodium hydroxid + 5% sodium chlorid	0.96	0.88	0.90	1.43	2.19	3.79	0.38	4.30	3.50	2.35	1.00
F.	Coca's solution	0.84	0.78	0.64	1.04	2.01	1.92	0.17	2.08	1.02	0.40	0.41
G.	10% alcohol	0.80	0.80	0.62	0.72	1.98	1.44	0.16	2.20	1.17	0.32	0.40
H.	70% alcohol	0.92	0.60	0.60	0.50	1.72	0.63	0.10	0.30	0.81	0.26	0.61
I.	95% alcohol	0.78	0.58	0.40	0.40	1.40	0.54	0.04	0.24	0.81	0.22	0.25

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C.	10% sodium chlorid	0.23	0.51	0.63	0.85	1.59	1.69	0.01	1.24	....	0.21	0.31
D.	0.5% sodium hydroxid	0.35	0.64	0.70	1.63	2.07	4.44	0.20	5.09	4.84	3.07	0.97
E.	0.5% sodium hydroxid + 5% sodium chlorid	0.29	0.62	0.69	1.21	1.89	3.52	0.20	4.06	3.22	2.22	0.91
F.	Coca's solution	0.32	0.56	0.43	0.71	1.73	1.60	0.02	1.80	0.67	0.29	0.34
G.	10% alcohol	0.21	0.80	0.44	0.56	1.61	1.15	0.01	2.03	0.82	0.21	0.31
H.	70% alcohol	0.11	0.60	0.41	0.31	1.28	0.34	....	0.13	0.46	0.15	0.53
I.	95% alcohol	....	0.26	0.23	0.40	0.92	0.25	....	0.07	0.46	0.11	0.17

TABLE 4.—*Method of Tabulating Results of Skin Reactions with Horse Dander*

	Solvent	Total Nitrogen, Mg. per C.c.	Nonprotein Nitrogen, Mg. per C.c.	Names of Subjects: M. J. M.	C. F.	Liv.	R. D.	W. D.	M. M.	P. R.
A.	1% sodium chlorid	2.02	....	+++	++++	+++	+++	++++	+	++++
B.	5% sodium chlorid	2.01	0.30	+++	++++	++++	+++	++++	++++	+++
C.	10% sodium chlorid	1.89	....	+++	++++	++++	++++	++++	++	++++
D.	0.5% sodium hydroxid	2.40	0.33	0	++	+++	++++	+++	++++	0
E.	0.5% sodium hydroxid + 5% sodium chlorid	2.19	0.30	0	+	+++	++++	+++	+	0
F.	Coca's solution	2.01	0.28	+++	0	++++	++++	++++	+++	+++
G.	10% alcohol	1.98	0.37	+++	+++	++++	++++	++++	+++	++++
H.	20% alcohol	1.72	0.44	0	0	0	+++	+	++	0
I.	95% alcohol	1.40	0.48	0	++	0	++	+	0	0

The skin tests reported were made by a single observer by the usual cutaneous scratch method on the arm or back of the person tested. A complete set of solutions of one material was tested at one time, in order to minimize the variations in reactions of the individual as much as possible. The markings indicating the reactions have the following significances: Zero denotes no reaction; + a questionable reaction; + + a distinctly positive but weak reaction; + + + a good positive reaction, and + + + + a strongly positive reaction, usually more than 1.5 cm. in diameter. In addition, it might be stated that no skin manifestation was called a reaction unless it showed definitely the pseudopods that we have learned to associate with the typical allergic skin reaction (Table 4).

TABLE 5.—*Summary of Skin Reactions Obtained by Using Various Extracting Fluids*

	A	B	C	D	E	F	G	H	I
Cabbage.....	2/4	4/4	3/4	0/4	2/4	1/4	0/4	2/4	1/4
Banana.....	2/2	2/2	2/2	2/2	2/2	2/2	1/2	1/2	1/2
Rye.....	2/3	3/3	3/3	1/3	0/3	2/3	2/3	0/3	0/3
Pea.....	1/1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1
Horse dander...	7/7	7/7	7/7	5/7	5/7	6/7	7/7	3/7	3/7
Chicken.....	1/1	1/1	1/1	1/1	1/1	0/1	0/1	1/1	1/1
Dog hair.....	2/4	3/4	3/4	1/4	3/4	1/4	3/4	1/4	1/4
Peanut.....	3/3	3/3	2/3	2/3	2/3	3/3	3/3	1/3	1/3
Salmon.....	1/2	1/2	1/2	0/2	2/2	1/2	1/2	0/2	0/2
Wheat.....	3/3	3/3	3/3	0/3	3/3	2/3	2/3	2/3	2/3
Walnut.....	3/4	3/4	3/4	1/4	2/4	3/4	1/4	0/4	0/4
Totals.....	27/34	31/34	29/34	14/34	22/34	21/34	20/34	11/34	10/34

Table 5 is a summary of the results obtained by using the solutions described above in the protein skin tests. In this table, the denominator of each fraction indicates the number of individual tests with each solution for each substance, and the numerator the number of positive skin reactions obtained. Thus, the total number of tests for each solution of each substance was thirty-four. It is seen on consulting this table that, from the clinical side, the most efficient extractives were Solutions A (1 per cent. sodium chlorid), B (5 per cent. sodium chlorid) and C (10 per cent. sodium chlorid), which gave the highest total number of positives, in spite of the fact that they do not contain either as much total nitrogen or protein nitrogen by difference as do Solutions D and E. This, of course, is probably explained by the fact that the alkali content in Solutions

D and E is sufficient to hydrolyze the proteins or other active substances present with consequent loss of activity. The least effective extractive proved to be the 95 per cent. alcohol, as would be expected. However, inspection of the table shows that although alcohol in this concentration is a very poor solvent for proteins, yet the considerable number of reactions obtained with many of the extracted materials indicates that some active substances, whether proteins or not, are extracted by this solvent.

SUMMARY AND CONCLUSIONS

1. Owing to our fragmentary knowledge of protein chemistry as applied to allergy, further research into the chemical nature of proteins and their clinical application in allergy is needed.

2. A systematic study of the actions of various chemical solvents in extracting substances capable of producing skin reactions indicates that the solvent known to be most effective in extracting the natural proteins without denaturization, namely, salt solution up to 10 per cent., is also most effective in obtaining skin reacting substances.

3. Neither the total nitrogen content nor the so-called protein nitrogen content by difference can be used to foretell the activity of a solution.

4. Ninety-five per cent. alcohol, although not a protein solvent, extracts substances that produce allergic skin reactions.

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