LXIX. ON THE CARDIAC, HAEMOLYTIC AND NERVOUS EFFECTS OF DIGITONIN.

By FRED RANSOM.

*From the Pharmacological Laboratory of the London (Royal Free Hospital)
School of Medicine for Women (University of London).*

(Received July 19th, 1922.)

DIGITONIN, a sapo-glucoside found in *Digitalis purpurea*, has certain well-known properties—it causes haemolysis, forms a combination with cholesterol and some other sterols and has a marked action upon cardiac muscle.

The objects of the present investigations were (1) to compare the effects of certain allies of cholesterol upon the cardiac and haemolytic actions of digitonin and (2) to ascertain whether the activities of a digitonin solution are modified by shaking with an indifferent adsorbent.

The particular digitonin used was prepared and carefully purified by Mr J. A. Gardner, whom I desire to thank for his kind help.

In the first place it was necessary to measure both the cardiac and the haemolytic activity of the preparation.

Haemolysis. Graduated amounts of digitonin, in each case dissolved in 4 cc. of 0.95 % NaCl solution, were placed in a series of test tubes and 1 cc. of a 10 % dilution of defibrinated ox blood in physiological salt solution was added to each tube, so that all reactions took place in 5 cc. of fluid. The tubes were left standing in the laboratory for about 18 hours and then the condition of their contents was noted, *i.e.* whether haemolysed or intact. In Table I the results of such a test are set out. Repeated experiments showed that the minimum quantity of the digitonin which would, under the above conditions, completely luke 0.1 cc. of ox blood was from 0.0032–0.0036 mg., corresponding to a dilution of the digitonin of from 1 : 156,000 to 1 : 139,000. With cat's blood the minimum complete laking dose was rather less than 0.004 mg.

Table I. *Haemolytic power of Gardner's digitonin.*

Digitonin 0.004 %	NaCl 0.95 %	Ox blood 10 %	Dilution of digitonin	Amount of digitonin	Condition after 20 hrs. at room temp.
0.4 cc.	3.6 cc.	1 cc.	1 : 312,500	0.0016 mg.	Intact
0.5	3.5	1	1 : 250,000	0.0020	Very slight haemolysis
0.6	3.4	1	1 : 208,000	0.0024	Slight haemolysis
0.7	3.3	1	1 : 178,577	0.0028	Nearly haemolysed
0.8	3.2	1	1 : 156,250	0.0032	Very nearly haemolysed
0.9	3.1	1	1 : 139,000	0.0036	Completely haemolysed
1.0	3.0	1	1 : 125,000	0.004	„ „

Evaporation to dryness on a water-bath of a digitonin solution, whether in water or 90 % alcohol, did not affect the haemolytic power (Table II) but the presence of NaCl in hypertonic solution increased the resistance of the red cells to haemolysis by digitonin (Table III), as was previously observed by Luger [1921] for saponin.

Table II. *Digitonin solution evaporated to dryness and redissolved.*

Digitonin	NaCl	Ox blood	Condition after
0.004 %	0.95 %	10 %	18 hours
0.4 cc.	3.6 cc.	1 cc.	Trace of haemolysis
0.6	3.4	1	Partial "
0.8	3.2	1	Complete "
0.9	3.1	1	" "

Table III. *Protective action of hypertonic NaCl.*

Digitonin	NaCl	10 % blood	Condition after 18 hours at room temp.
0.004 mg.	4 cc. .95 %	1 cc.	Complete haemolysis
0.004	4 cc. 2.5 %	1	Partial "
0.004	4 cc. 6.0 %	1	No "
0.008	4 cc. 5.0 %	1	Complete "

Fig. 1. Frog's heart perfused, with Ringer's fluid. At arrow perfusion changed to Ringer + 0.004 % digitonin.

Cardiac action. Solutions of the digitonin in oxygenated Ringer's fluid were perfused through frogs' hearts *in situ* from a cannula inserted into the inferior cava, the fluid passing at a constant and moderate pressure through the heart and escaping by the cut aorta. A number of such perfusions showed that 0.0001 % of digitonin had very little or no effect; 0.001 % produced increase in the beat followed by rise of tone, diminution of diastole and finally stoppage or nearly so; 0.004 % produced the same effects somewhat more quickly (Fig. 1). Evaporation of a watery solution to dryness on a water-bath and redissolving in Ringer's fluid did not affect the activity. Perfusion of a frog's heart with a Ringer's fluid modified by omitting Ca quickly causes diminution of efficiency; if now, in the continued absence of Ca, 0.001 % digitonin is added to the perfusing fluid the normal efficiency is first partly restored and then the characteristic effect of digitonin is developed.

It is well known that digitonin treated with cholesterol loses its haemolytic power, it seemed therefore of interest to ascertain in how far bodies more or less closely allied to cholesterol such as phytosterol, β -cholestanol, coprosterol and pseudo-coprosterol affect the action of digitonin upon the blood and upon the heart.

EXPERIMENTS WITH SUBSTANCES ALLIED TO CHOLESTEROL.

Method. A solution containing 0.004 % (or other percentage) of digitonin in 90 % alcohol was mixed with an equal quantity of a solution of the same strength of one of the cholesterol allies, also in 90 % alcohol. The mixture was evaporated to dryness on a water-bath and the dry residue taken up in so much Ringer's fluid as would give a possible concentration of digitonin and the sterol equal to that of the original alcoholic solution. This solution of the dry residue was filtered and the filtrate tested for its haemolytic power and cardiac effect. The results obtained with phytosterol, β -cholestanol, and coprosterol were identical—in each case both the haemolytic effect and the cardiac action were either cut out or greatly diminished (haemolytic effect, Table IV). On the other hand, the mixture digitonin + pseudo-coprosterol retained both its haemolytic and cardiac activity.

Table IV. *Effect of phytosterol, β -cholestanol, coprosterol and ψ -coprosterol.*

Possible concentration of digitonin 0.004 %.					
	Solution of residue	NaCl 0.95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
Phytosterol or β -Cholestanol or Coprosterol ψ -Coprosterol	1 cc.	3 cc.	1 cc.	1 : 125,000	No haemolysis
	2	2	1	1 : 62,500	"
	4	—	1	1 : 31,250	"
	1	3	1	1 : 125,000	Complete haemolysis

Control for above: Digitonin 0.004 % in 90 % alcohol evaporated to dryness and taken up in NaCl, so as to give a possible 0.004 % digitonin.

	Solution of residue	NaCl 0.95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
	0.6 cc.	3.4 cc.	1 cc.	1 : 208,333	No haemolysis
	0.8	3.2	1	1 : 156,250	Partial haemolysis
	1.0	3	1	1 : 125,000	Complete "

When stronger solutions of digitonin were mixed with correspondingly increased amounts of phytosterol, β -cholestanol or coprosterol the protection against haemolysis and cardiac action was still complete (Table V).

Table V. *Protective action of phytosterol, β -cholestanol or coprosterol.*

Possible concentration of digitonin 0.1 %.				
Solution of residue	NaCl 0.95 %	Ox blood 10 %	Dilution of digitonin	Condition after 18 hrs. at room temp.
0.5 cc.	3.5 cc.	1 cc.	1 : 10,000	No haemolysis
1.0	3.0	1	1 : 5,000	"
4.0	—	1	1 : 1,250	"

If the haemolytic test was carried out with mixtures containing a certainly haemolytic dose of digitonin and a gradually diminishing amount of phytosterol, β -cholestanol or coprosterol, the effect of these bodies in diminishing and finally extinguishing the haemolytic effect of the digitonin (Table VI) was perhaps still more strikingly in evidence.

Table VI. *Protective action of phytosterol.*

Digitonin 0.01 %	Phytosterol 0.01 %		Cat's blood 10 %	Condition after 20 hrs. at room temp.
0.5 cc.	+	0.5 cc.	1 cc.	No haemolysis
0.5	+	0.4	1	"
0.5	+	0.3	1	"
0.5	+	0.2	1	"
0.5	+	0.1	1	Some haemolysis
0.5	+	0.05	1	Complete haemolysis
0.5	—	—	1	"

In all the above experiments the solutions used were of equal percentage for the digitonin and the cholesterol allies; now the molecular weight of Gardner's digitonin is 1202, that of cholesterol and its isomer phytosterol is 386, so that when equal percentages by weight are used 1 phytosterol corresponds molecularly to 3.1 of digitonin. It was found that 0.31 cc. of a 0.01 % solution of digitonin (0.0031 mg.) will very nearly or sometimes completely haemolyse 0.1 cc. of ox blood. If the relationship between digitonin and cholesterol in the digitonin-cholesteride is monomolecular [Windaus, 1910], then 0.31 cc. of a 0.01 % solution of digitonin should be inactivated by 0.1 cc. of a 0.01 % solution of phytosterol or β -cholestanol or coprosterol (since the difference between the molecular weight of phytosterol, 386, and that of β -cholestanol or its isomer coprosterol, 388, is too small to be of importance in the conditions of the experiment); moreover multiples of these quantities should also be inactive. On the other hand, if the mixtures contain relatively an excess of digitonin more or less haemolysis should occur. In Table VII the results of an experiment carried out on these lines are shown. It will be seen that when molecular equivalents were mixed the neutralisation of the haemolytic action of the digitonin was complete, even when multiples of the minimum haemolysing dose were employed; when, however, the digitonin was relatively in excess haemolysis occurred and was most marked in the tubes containing the smallest relative proportion of the sterol.

As regards the cardiac activity it was found that the effect upon the heart ran parallel with that upon the red cells, the non-haemolytic mixtures had no cardiac effect and when the haemolytic action was not completely cut out there was also some cardiac effect. The results recorded in Table VII are strongly reminiscent of what occurs when diphtheria or other toxins are mixed with their respective antitoxins, and might perhaps be explained as simple adsorption phenomena (see below) were it not that other evidence points to a definite chemical union between digitonin and cholesterol. Moreover the fact that pseudo-coprosterol does not interfere with either the

haemolytic or the cardiac action of digitonin strongly supports the chemical view. In view of the chemical relationship between cholesterol and cholalic acid a series of experiments was made with this substance on the same lines as those recorded above, using a 0.01 % solution of the acid with 0.01 % of digitonin, but no evidence was obtained which would indicate that cholalic acid has any influence either upon the haemolytic or the cardiac action of digitonin.

Table VII.

All reactions in 5 cc. of fluid.				Condition after 18 hrs. at laboratory temp.	
0.01 % sol. of coprosterol	0.01 % sol. of digitonin	Cat's blood 10 %			
0.1 cc.	0.31 cc.	1 cc.	Molecular equivalents	{	No haemolysis
0.2	0.62	1			"
0.4	1.24	1			"
1.0	3.1	1			"
0.05	0.31	1	Digitonin in excess	{	No haemolysis
0.1	0.62	1			Trace of haemolysis
0.2	1.24	1			Partial "
0.5	3.1	1			Complete "
0.2	3.1	1	Digitonin in excess, constant, coprosterol varied	{	Completely haemolysed
0.4	3.1	1			" "
0.6	3.1	1			Nearly "
0.8	3.1	1			No haemolysis
—	0.1	1	Controls for digitonin	{	No haemolysis
—	0.2	1			Some "
—	0.3	1			Complete haemolysis
—	0.4	1			" "
—	0.5	1			" "

ADSORPTION EXPERIMENTS.

The importance of adsorption in many pharmacological problems suggested experiments to ascertain if the solutions of digitonin are affected by indifferent adsorbents. To this end experiments were carried out with animal charcoal, kaolin and starch.

Method. 50 cc. of 0.004 % solution of digitonin in distilled water were gently shaken with 2 g. of purified animal charcoal (washed and dried) or kaolin or starch for 30 mins., then filtered and to the filtrate salts to make Ringer's fluid were added; this isotonic solution was then tested as to its haemolytic and cardiac activities.

Charcoal. The digitonin solution before treatment with charcoal, but with salts for Ringer's solution added, caused haemolysis as usual (0.8 cc. completely); after charcoal 5 cc. had no haemolytic effect. As to cardiac action the solution which had been treated with charcoal had no effect, but when the perfusion fluid was changed to the digitonin solution untreated with charcoal the usual digitonin effect appeared at once. The charcoal remainder was shaken with 25 cc. 90 % alcohol for 30 mins., the alcohol evaporated and the residue taken up in 50 cc. Ringer's fluid, this solution caused haemolysis and had a marked cardiac action. It is evident that animal charcoal very effectively adsorbs digitonin. If the period during which the charcoal

was applied or the amount of charcoal used was diminished the removal of the digitonin was not so complete.

Starch. In this case the digitonin solution was 0.001 %. This solution perfused through a frog's heart gave rise to increased systole and rise of tone, after treatment with starch the filtrate showed only a slight remnant of digitonin action. As to haemolysis, the results of the test are given in Table VIII and show that whereas before treatment with starch 1 cc. of the digitonin solution caused complete haemolysis, after starch 5 cc. did not do so. Digitonin then is adsorbed by starch and the filtrate from the starch is found to have lost both in haemolytic and cardiac activity.

Table VIII.

Before starch 1 cc. of 0.001 % digitonin gave complete haemolysis					
After	"	1	"	"	no haemolysis
"	"	2	"	"	"
"	"	3	"	"	trace only
"	"	5	"	"	nearly complete haemolysis

Kaolin. In this case also the digitonin solution used was 0.001 %. Before treatment with kaolin 1 cc. completely haemolysed 0.1 cc. of ox-blood, after kaolin 5 cc. caused only partial haemolysis (Table IX). As to cardiac action, before kaolin the solution gave a characteristic digitonin effect, after kaolin there was still some evidence of digitonin effect but much less marked. Clearly, then, kaolin adsorbs digitonin from watery solution.

Table IX.

Before kaolin 1 cc. of digitonin sol. gave complete haemolysis					
After	"	2	"	"	no haemolysis
"	"	4	"	"	trace of haemolysis
"	"	5	"	"	incomplete haemolysis

At first glance it would appear as though of the three adsorbents animal charcoal were the most effective but much stress cannot be laid upon this point, since no attempt was made to insure the same number and size of particles, *i.e.* surface area, in the amounts of each of the adsorbents. Nevertheless there are good grounds for believing that charcoal occupies a peculiarly favourable position among adsorbents [Michaelis and Rona, 1920].

Windaas founded his method of estimating cholesterol by means of digitonin upon his discovery that cholesterol forms with digitonin a stable compound which is not haemolytic and dissolves with difficulty in alcohol and upon the fact that cholesterol esters [Hausmann, 1905] do not form such a compound. He considered the digitonin-cholesteride to be an addition product and not an ester and gave its formula as $C_{55}H_{94}O_{28}$ (digitonin) + $C_{27}H_{46}O$ (cholesterol), regarding it as a combination of one molecule of cholesterol with one of digitonin without loss of water. The results obtained in the present investigation are confirmatory of this view, since they show that when phytosterol, coprosterol or β -cholestanol are mixed in monomolecular proportions with digitonin the latter loses completely its haemolytic power,

whereas, if the mixtures contain less than this proportion of the sterol, more or less haemolysis takes place according to the degree of deficiency.

But now the very interesting question arises as to how the cardiac action of digitonin is affected. In the first place it may be noted that the effect of phytosterol, β -cholestanol or coprosterol upon the cardiac action runs strictly parallel to their effect upon the haemolytic power of digitonin—with monomolecular proportions both the haemolytic and the cardiac actions were cut out, whereas in mixtures containing a deficiency of sterol the more nearly the haemolytic action was neutralised the less was the cardiac effect and *vice versa*. Further, pseudo-coprosterol which does not prevent haemolysis, *i.e.* presumably does not combine with digitonin, did not affect the cardiac action either. These facts suggest that the action of digitonin upon the heart muscle depends upon the same factor as that which determines the haemolytic effect, *viz.* that there is present in cardiac muscle a body resembling or identical with cholesterol or coprosterol, etc., and that this sterol is at any rate in part free and not esterified. Moreover it has so important a function to perform in the muscle that its withdrawal as digitonin-sterol profoundly affects the physiological activity of the muscle cell. This supposition receives important confirmation from the fact that pseudo-coprosterol, which does not form a digitonide and does not affect haemolysis, has no effect upon the cardiac action of digitonin.

To show that the heart muscle probably does fix digitonin, the following experiment was carried out; a kitten was killed by pithing the brain and bleeding, the blood being collected and defibrinated. The heart was removed, washed free from blood, and the coronary vessels washed out with Ringer. The excised heart (7 g.) was pounded to as smooth a paste as possible, then well mixed with 50 cc. 0.002 % digitonin in Ringer and the mixture placed in the ice-chest. After 20 hours the mixture was centrifuged, the liquid poured off and divided into two parts, one of which was used to test haemolysis (Table X); with the other a frog's heart was perfused, with the result that both the haemolytic and the cardiac effects were markedly lessened.

Table X.

Heart emulsion + digitonin 0.002 %	Ringer	Kitten's blood 10 %	After 20 hrs.
0.8 cc.	4.0 cc.	0.2 cc.	Intact
1.0	3.8	0.2	"
2.0	2.8	0.2	"
5.0	—	0.2	"
Digitonin sol. 0.002 %			
0.8 cc.	4.0 cc.	0.2 cc.	Partly haemolysed
1.0	3.8	0.2	Nearly completely
2.0	2.8	0.2	Completely

It may be recalled that many of the saponins, including digitonin, are powerful fish poisons, acting by paralysing the C.N.S., but that the cholesterol-saponide is non-toxic as well as non-haemolytic. In like manner saponin,

e.g. agrostemma saponin, found in the corn cockle, when it causes poisoning in human beings does not kill by its action upon the blood but by its effect upon the C.N.S. and upon the heart. On the other hand, tetanus toxin, besides its characteristic action upon the C.N.S., has definite haemolytic powers, indeed tetanus toxin is usually regarded as a mixture of tetanospasmin and tetanolysin. Cholesterol neutralises tetanolysin without diminishing the specific action of tetanospasmin. So the question arises; is the central nervous effect of saponin due to the same factor as enables it to cause haemolysis and alteration in cardiac muscle or do the conditions resemble those in tetanus toxin? To throw light upon this point the following experiment was carried out. A young rabbit was killed by a blow on the neck, the carotids were then cut and the blood collected and defibrinated; brain removed (6.7 g.), rubbed

Fig. 2. Frog's heart perfused with Ringer's fluid. At arrow perfusion changed to emulsion of rabbit's brain in Ringer + 0.004 % digitonin. Compare with Fig. 1.

to a smooth paste in a mortar and then thoroughly mixed with 50 cc. of a 0.004 % solution of digitonin in Ringer's fluid; the emulsion was placed in the ice-chest for 20 hours; then well centrifuged, the supernatant fluid poured off and divided into two parts, one of which was used to perfuse a frog's heart, the other to test the haemolytic power. Table XI shows the results of the haemolytic test, the perfusion tracing is shown in Fig. 2. It will be seen at once that the digitonin-brain emulsion has no haemolytic power, at least not up to 5 cc. which is very much more than the ordinary haemolytic dose, and further the heart tracing shows merely a trace of digitonin action.

Table XI. *Protective action of brain substance.*

Possible concentration of digitonin, 0.004 %.			
Brain + digitonin	Ringer	Ten rabbits' blood	After 18 hrs.
0.6 cc.	4.2 cc.	0.2 cc.	Intact
0.8	4.0	0.2	"
1.0	3.8	0.2	"
2.0	2.8	0.2	"
5.0	—	0.2	"
Digitonin 0.004 %			
0.8 cc.	4.0 cc.	0.2 cc.	Completely haemolysed

Considered in conjunction with the previous experimental evidence, these results appear to justify the conclusion that the action of saponin in causing narcosis in fishes and paralysis of the C.N.S. in man is an analogous phenomenon to digitonin haemolysis and digitonin cardiac action, *i.e.* there is free and unesterified in the brain a body allied to or identical with cholesterol, phytosterol, etc., which unites with digitonin. Moreover the function which this cholesterol-like body has to perform is of such importance that when it is inactivated by combination with digitonin (saponin) serious central nervous symptoms ensue.

Fig. 3. Two frogs' hearts perfused with Ringer's fluid. *A*, at arrow perfusion fluid changed to emulsion of cat's brain in Ringer + 0.001 % strophanthin. *B*, at arrow perfusion fluid changed to Ringer + 0.001 % strophanthin. In each case the break in the tracing represents an interval of 15 mins.

Kobert [1904] has shown that the toxic action of saponin as well as the cardiac effects is abolished by the combination of saponin with cholesterol, and further that the extreme toxicity of sapotoxin solutions for fishes is entirely lost in the cholesterol-sapotoxin combination.

The effect of digitonin is also neutralised by adding milk to the digitonin solution.

It is interesting to note that strophanthin, which is a glucoside but not a saponin, does not lose its cardiac action when brain emulsion is added to the strophanthin solution (Fig. 3).

SUMMARY.

Monomolecular combinations of digitonin with phytosterol, coprosterol, β -cholestanol are non-toxic for red blood cells and for the frog's heart; any excess of digitonin beyond this proportion can be detected by the action of the solution upon red cells and upon the heart. Pseudo-coprosterol does not effect the activity of digitonin. Digitonin mixed with an emulsion of brain substance loses its toxicity for red cells and for the heart.

It is suggested that the toxic action of digitonin upon red blood cells, cardiac muscle cells and cells of the central nervous system (fishes) is essentially the same—it attacks a substance allied to or identical with cholesterol which is present free and not esterified in these various cells. This free sterol has in each case so important a function to perform that its inactivation by combination with digitonin leads to a profound modification of the physiological activities of the respective cells.

Digitonin is readily adsorbed from solution in water (*e.g.* 1 : 25,000) by animal charcoal, kaolin or starch; the adsorption by charcoal appears to be particularly complete.

Cholalic acid has no effect upon the action of digitonin.

REFERENCES.

- Hausmann (1905). *Beiträge*, 6, 567.
Kobert (1904). *Saponinsubstanzen*, Stuttgart.
Luger (1921). *Biochem. Zeitsch.* 117, 145.
Michaelis and Rona (1920). *Biochem. Zeitsch.* 102, 268.
Windaus (1910). *Zeitsch. physiol. Chem.* 65, 110.