PHYSIOLOGICAL ASPECTS

of

MILK EJECTION

A THESIS

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I N T R O D U C T I O N

During the latter part of the last century, a number of physiologists conceived the idea that the functions of the mammary growth and milk secretion were under the control of the nervous system. As a consequence, many experiments were carried out with the object of elucidating the role of the nervous system in lactation; these culminating in the classic experiments of Ribbert. In the year 1898, this man succeeded in transplanting mammary tissue from the inguinal region of the guinea pig to an area behind the ear, thus demonstrating that the mammary gland could grow, and to a limited extent function, independent of nervous connections.

Attention was then focused on the possibility of a purely endocrine control of the mammary gland, a concept that has given rise to much valuable knowledge by virtue of the experimentation it has stimulated. However a third phase in the history of research into mammary gland function is now being entered upon. As with a general tendency of investigations in endocrinology as a whole, integrations are being sought between endocrine and nervous mechanisms.

In 1941 Ely and Petersen postulated the neuro-endocrine theory of milk let-down. This theory suggested that the discharge of milk from the mammary

gland, as distinct from milk secretion, was brought about by the release of a humonal substance from the posterior pituitary into the blood stream, in response to nervous impulses reaching the pituitary from the mammary gland. At this stage there was but limited evidence suggesting the implication of the pituitary gland in such a phenomenon, the nerve supply of the udder was incompletely understood, whilst the existence and mechanism of myoepithelial cell contraction were subjects only for conjecture. In the succeeding ten years, data has accumulated concerning these three points, while increasing recognition has been given to the distinction between milk secretion and discharge. The evidence so adduced has served to support the neuro-endocrine theory of milk let-down.

Petersen (1944) has further suggested that a lack of persistency in milk yield may be related to an imperfect functioning of the neuro-endocrine relationship involved in milk discharge. Petersen's theory, while lacking the support of precise experimental findings, serves to explain many observations regarding the lactational behaviour of both dairy animals and lactating humans, and thus is one that may be capable of directing research into highly profitable fields.

Turner and Cooper (1941) devised an assay technique for the milk ejection hormone, using the lactating rabbit as an assay animal. However, their

assay depended upon minimal responses - a result that does not necessarily indicate normal ejection responses. Ely and Petersen (1941) studied the response of the lactating cow, but this method is unsatisfactory because of the difficulty in interpreting the milk ejection curve of a cow. This curve is determined partly by the sphincter tension and size, and partly by the volume of the milk cistern; complications which obscure the interpretation of the immediate effects of the injected material. The technique of Whittleston (1952) using the lactating sow, allows of a new approach to the problem of milk ejection. The sow has numerous advantages for such a study. It has no expansive milk cistern (Turner 1952), its "sphincter" does not require a marked pressure difference across it before the milk will flow (Turner 1952), it may be handled with ease, and is of small commercial value compared to the larger farm animals.

Using the technique of Whittleston, a study has been made of the phenomenon of milk ejection in the sow with the following objects in view :

(I) To develop the technique to the level of an accurate assay procedure.

(2) To elucidate factors influencing the let-down response.

(3) To further knowledge concerning the efficiency of milking and of milk production in dairy animals, bearing in mind the concept that the milk production of an

animal may be limited by the sub-optimal functioning of the let-down mechanism.

Such a long range object as the last involves a detailed knowledge of the physiology of the posterior pituitary, the mammary gland, and the nervous system which relates them. The first two chapters of this thesis review the literature and summarize the available knowledge in these fields, knowledge without which a critical approach to the problems of milk ejection cannot be readily undertaken. The remaining chapters are an exposition of the experiments performed, and the results and conclusions drawn from them.

P A R T I

A - REVIEW OF LITERATURE

THE MECHANISM OF MILK LET-DOWN

The Development of the Neuro-Endocrine Theory

Among early ideas regarding milk formation, based upon observation only, was the belief that the act of milking resulted in the formation of milk. An illustration of this notion is afforded by a quotation from the text of Judkins College text book (1924) :

> "The udder contains only a small amount of milk, usually between a pint and a quart, when one starts milking. This is found in the four milk cisterns. The enlargement of the udder which occurs before milking is doubtless due to the storing up of the ingredients out of which the milk is to be made. After the first milk is drawn, the cow, by nervous tensions, tightens up the muscles located at the points where the ducts branch off and simply stops making milk until she is ready to do so. When that time comes, the gland lobules and their contents, in some mysterious way, put the stored ingredients together into milk which trickles down the ducts to the cistern, thence it passes to the teat canal & the milker squeezes out. For the most part therefore, milk is really made during the milking process. A cow killed just before milking will be found to have no milk in the udder except that present in the milk cisterns."

This idea, was no doubt, based upon the phenomena of "milk let-down" a process whereby the udder enlarges, becomes turgid and shows signs which might be taken as evidence of active secretion.

By 1900, information was available indicating that direct nervous innervation of the mammary gland was not responsible for its physiological function. This fact was well demonstrated by two classic experiments. Routh (1896) observed that lactating women suffering a severed spinal cord exhibited normal lactogenesis, while Galtz and Ewald (1896) severed the spinal cords of various animals without impairing mammary function. Eckhard in 1877 had noted continued lactation from a gland whose nervous supply was severed, while in 1898 Hugo Ribbert succeeded in transplanting two mammary glands of guinea pigs to the skin at the back of the ear of sister animals, where laction was initiated after parturition.

In 1915 Gaines stated that the processes of milk ejection and milk secretion are separate and distinct entities. The conclusion reached by Gaines was based upon the observation that the ejection of milk in a goat was co-incident with a high intra gland pressure, and that low pressure periods occurred between high pressure periods. That pressure is related to the rate of milk ejection was further

demonstrated by Tgetgel (1926). Measuring mammary pressure by manometer readings from the nipple, Tgetgel showed that the pressure gradually increased from one milking to the next as milk accumulated in the cistern. At the beginning of milking however, there was a sudden and very large increase of pressure, and then as milking proceeded the pressure gradually fell (Fig.1.1).

The theory that the sudden increase in pressure was due to a reflex secretion of milk is now discounted, primarily on the grounds that all the milk which is normally obtained at any one milking is already in the udder of the animal before milking begins (Gaines and Sanmann 1927).

Several explanations suggesting the cause of the sharp pressure rise recorded at the time of milking have been offered. Hammond (1936) suggested it was due to an erection of the udder and nipple, caused reflexly by stimulation of the nipple during the act of suckling or milking. This erection was reputed to put pressure on the milk contained in the ducts and alveoli, resulting in a marked increase in the cistern pressure. He suggested that afferent fibres carried the stimuli to a centre in the spinal cord from which the efferent nerves conducted the impulses to smooth muscle fibres, and perhaps the basket cells about the alveoli, running in conjunction

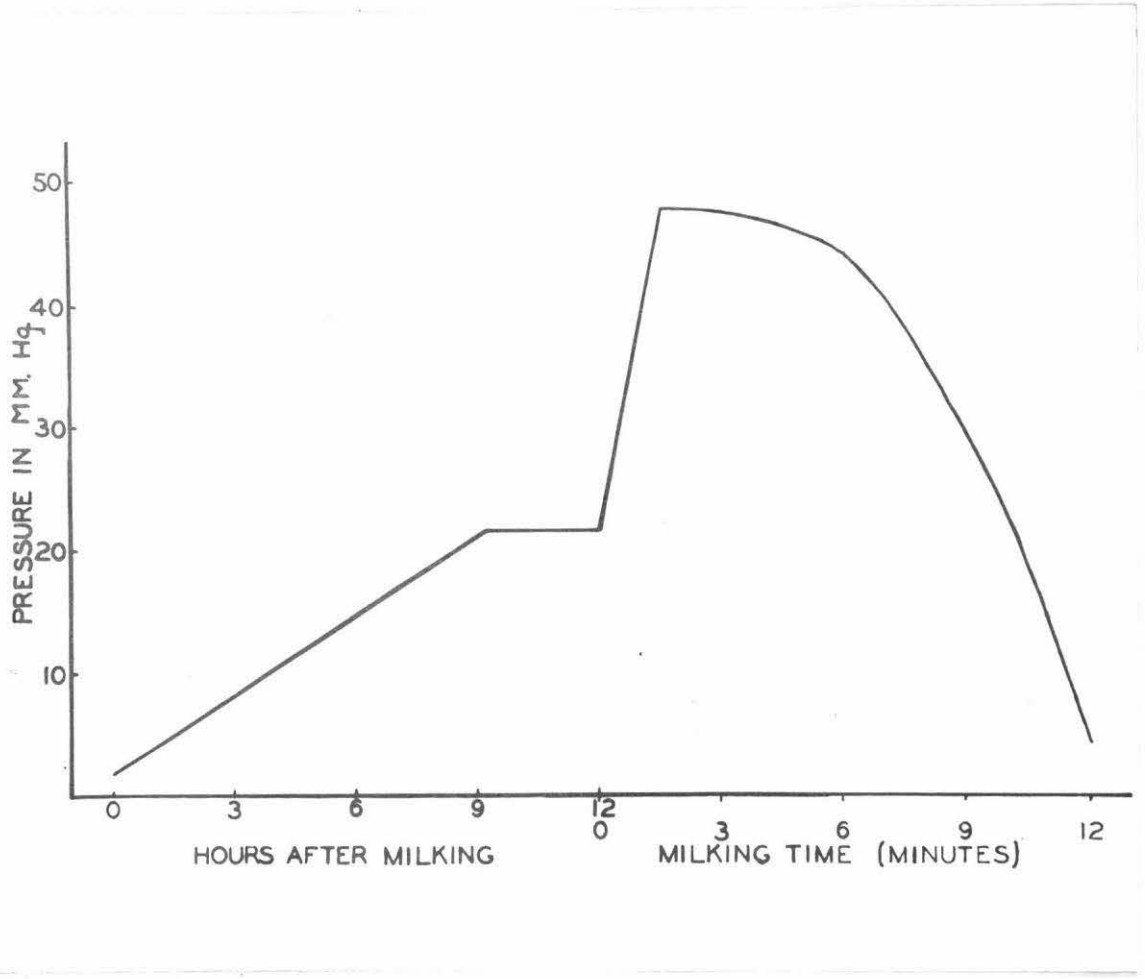


Fig.1.1 - Diagram of Milk Pressure changes in the Udder (from Tgetgel).

with or over veins, and by occluding the latter, caused accumulation of blood in the tissues of the udder and nipples. Gaines (1915) had supposed a reflex contraction of smooth muscle in the glands to be the cause of milk ejection, a view supported by Krupski (1925). Krzwanek and Bruggemann (1931) believed the contraction of "kabzellen" (basket cells covening the alveoli) to be the primary cause.

Meanwhile Ott and Scott (1912) has demonstrated that the injection of an extract of the posterior lobe of the pituitary into a lactating goat caused the discharge of milk from the mammary gland. In 1915 Gaines suggested that "pituitrin" (an extract of the posterior lobe of the pituitary) had a muscular action on the active mammary gland, causing a constriction of the milk ducts and alveoli with a consequent expression of milk. This action took place in the excised gland in the absence of any innervation as well as in the normal gland. Together with Sanmann in 1926 he postulated an hypothesis of milk secretion and discharge involving continuous intracellular milk formation, cellular discharge by membrane rupture to the duct system, and the subsequent removal of the milk by a contractile mechanism set in action by a nervous reflex initiated by the stimulus of milking. Hammond (1936) viewed the action of pituitrin as that of a galactogogue, a

suggestion refuted by Gaines and Sanmann. Turner and Slaughter (1930) showed that the injection of pituitrin permitted the removal from the udder milk that was otherwise unavailable, and were thus "inclined to the theory that pituitrin is not a galactagogue but rather acts on the mechanism normally effective during the milking process."

Thus there was dispute as to the nature of the let-down process. The American workers favoured the view that the phenomena was activated by stimulation of the teats, causing a release of a pituitary factor into the blood, the mammary glands being the target organ, while Hammond propounded the idea that the ejection of milk was brought about by a nervous reflex causing an engorgement of mammary tissue with blood.

Ribbert (1898) and others had previously demonstrated that the nervous system did not exercise a direct control over the combined effects of secretion and ejection. MacKenzie (1911) and McCandlish (1918) tried numerous drugs, several of which might be classed as nerve stimulants, and failed to produce a marked effect on the rate of secretion or ejection of milk. Both however noted that pituitrin produced a marked milk discharge effect. Canon and Bright (1931) sympathectomised a dog, and from its behaviour during lactation concluded that the autonomic nervous system

was essential to this function. They described the effect as a belated one, causing the mother to be indifferent to her young, while viscous creamy material accumulated in her mammae. Inglebrecht (1935) sectioned the spinal cords of ten rats between the last thoracic and first lumbar vertebrae, thus denervating the six posterior glands while permitting the six anterior ones to remain intact. Nursing young died when permitted access only to the posterior six glands, but when two of the anterior glands were suckled, all glands functioned normally. Selye et al (1930) found that nursing caused continued gland function in adjacent glands which were not nursed. Thus, save for the single experiment of Canon and Bright all results could be explained in terms of a sensory nervous system - pituitary interaction.

The position regarding the role of the posterior pituitary was rather confused. Smith (1932) and Houssay (1935) removed the posterior pituitary of the rat and dog respectively and found no inhibition of lactation after parturition. Yet in 1939 Gomez reported that lactating hypophysectomized rats could be maintained in lactation only by replacement therapy with both anterior and posterior pituitary extracts. Without the posterior lobe therapy the young seemed unable to obtain milk present in the gland.

By 1928 Kamn and his co-workers had effected a fairly complete separation of two active constituents

of the posterior lobe. They found one fraction to have an oxytocic action, and named this preparation "Pitocin". The other fraction was found to cause an increase in blood pressure - to this substance they gave the name "Pitressin".

Using Kamm's oxytocic preparation, and a cow in which the afferent nerve fibres to one half of the udder had been cut, Ely and Petersen (1941) found that let-down could be evoked by milking, or by posterior pituitary extracts, or conversely inhibited in both halves alike by adrenalin or fright. On the basis of these results Ely and Petersen suggested that the let-down of milk involved a neuro-hormonal arc. The theory was postulated that palpation of the teat, and possibly other external stimuli, were sources of sensory impulses reaching the central nervous system which in turn stimulated the posterior lobe to secrete "oxytocin" into the blood. This factor was thought to cause the observed increase in intraglandular pressure. In a similar manner fright, causing an inhibitory reflex, stimulated the production of adrenalin by the medulla of the supra-renals.

In the same year Turner and Cooper (1941) found pitocin to have approximately five times the let-down activity of pitressin. They suggested the presence of a separate milk ejection hormone present in both preparations, for the milk ejecting activity of pitressin was somewhat greater than could be

accounted for on the basis of oxytocic contamination of the pressor principle.

The presence of a milk ejecting principle in the blood of a cow stimulated to let down, was demonstrated independently by Petersen and Ludwick (1942), and by Peeters, Massart and Coussens (1947), a finding which lent considerable weight to Ely and Petersen's theory.

In a paper presented to the N.Z. Dairy Science Association in 1948, Whittleston reported that MacFarlane, in a private communication, had clearly demonstrated the existence of a network of myoepithelial cells surrounding the alveoli and ductules; entities which might well be responsible for the physical act of ejection. On this basis Whittleston rejected the hypothesis of Hammond that milk let-down was due to an erectile tissue mechanism, for MacFarlane's sections showed no evidence of the blood vessels necessary for this hypothesis. In 1949 Richardson was able to clearly identify these basket-like cells about the alveoli and ductules, so confirming MacFarlane's communication. Linzell (1952) published a paper reaffirming the conclusions postulated by Richardson regarding the identity, contractility and participation of these myoepithelial cells in the let-down reflex. Fig.(1.2).

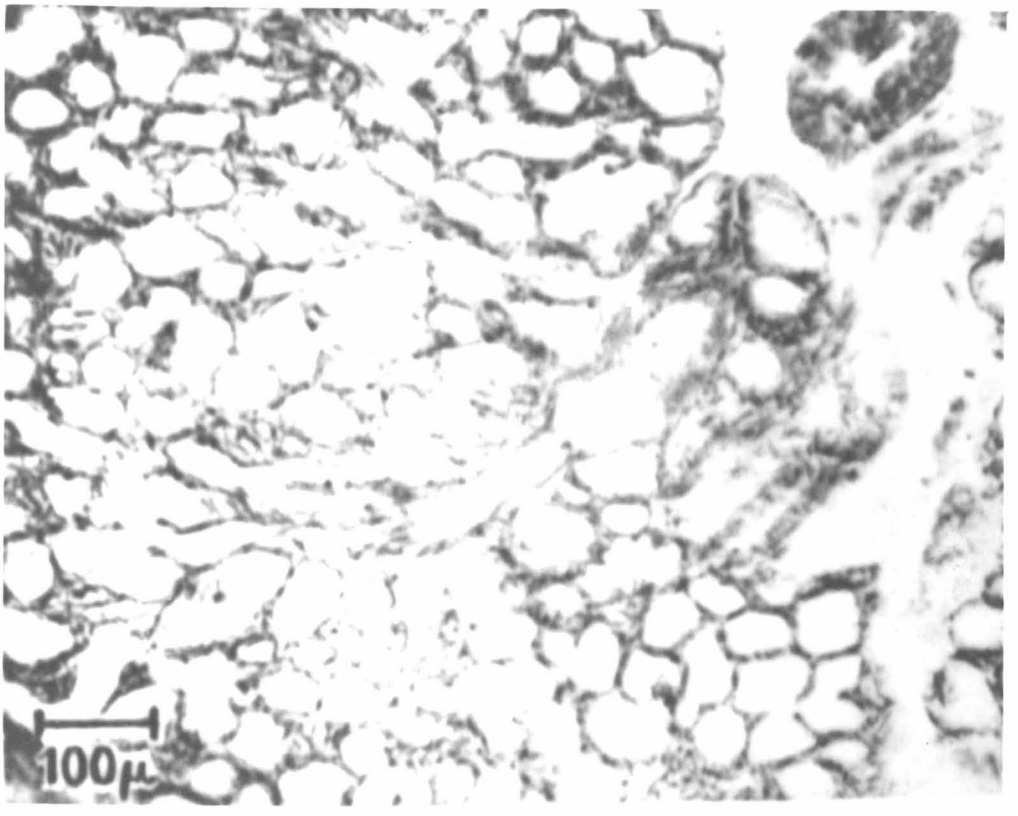


Fig.1.2 - General low power view showing abundance of myoepithelium. Background unstained. Tissue from a cat gland. Carvoy. Frozen section. (from Linzell)

The myoepithelial cells have been seen to run along the walls of the small ducts as well as about the alveoli. The significance of this fact is problematical in the recurring controversy concerning the active participation of the ducts in let-down. In support of the view that the contraction of myoepithelium might cause an opening and closing of these ducts are three pieces of evidence :

1. The obvious enlargement of duct size at let-down (Linzell 1952) (Fig.1.3).
2. Let-down in the sow and its cessation are rapid and valvelike in action.
3. If in the cow mammary pressure is built up by intraduct injections of saline until it exceeds the normal let-down pressure value, and posterior pituitary extract subsequently injected, there is a fall in pressure. If only alveoli were involved no change in pressure, in a negative direction at least, would be expected (Whittleston 1951).

Five important links in the chain of events leading to milk ejection have thus been established. In summarized form these are -

1. Pitocin is capable of eliciting a milk ejection response in both the normal and perfused gland.
2. The blood of a cow stimulated to let-down, contains a substance capable of evoking milk ejection in a perfused udder.

Fig.1.3a - Normal appearance of a distended gland showing evenly distributed alveoli as white dots and white milk filled ducts.

(From Linzell)

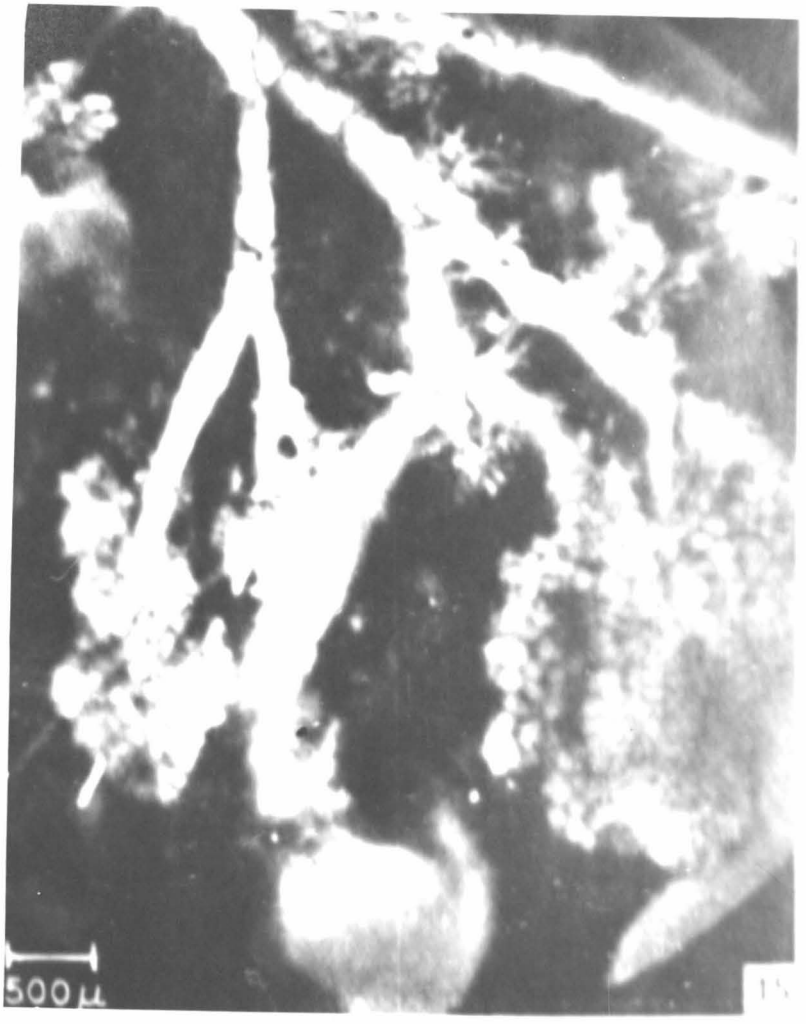


Fig.1.3b - The same field 2 minutes after applying 0.01 unit of Pitocin. Note that the majority of alveoli have been emptied of milk and can no longer be seen, whilst the ducts have become greatly extended.

(From Linzell)

3. The myoepithelial elements of the mammary gland contract under the action of pitocin and cause expulsion of milk.

4. The posterior pituitary appears to be an essential factor in the suggested neuro-endocrine arc.

5. The dennervation of the mammary gland prevents the let-down of milk.

The Role of the Autonomic Nervous System

The contractile elements of the mammary gland respond to a number of pharmacologically active substances, however this fact does not necessarily indicate that such substances are important physiologically. A summary of the effects ofmany of these substances on the milk ejection response and on blood flow is presented in Table I. Of these, particular attention has been focused on the response of the gland to adrenalin and to acetylcholine, since the effect of these drugs may be related to their respective sympathetico and parasympathetico mimetic properties. In view of the fact that the mammary gland is remarkably sensitive to both these drugs, the question of the role of the autonomic nervous system in milk let-down assumes considerable importance, for even if this system does not provide the normal mechanism of let-down, its influence may well be superimposed on, and thus modify the suggested endocrine control.

TABLE - 1

THE EFFECT OF VARIOUS PHARMACOLOGICALLY ACTIVE SUBSTANCES ON MILK EJECTION AND BLOOD FLOW IN THE MAMMARY GLAND

Substance	Amount	Species	Milk ejection response	Vascular response	Reference
Pitocin (oxytocic hormone)	10 units	Cow	Complete ejection	8-20% decrease in blood flow	Petersen, 1942.
	5-10 units	"	" "	No effect	Peeters et al 1952
	3 units	"	Incr. in cistern pressure	-	Peeters, Goussens & Oyaert, 1949
	0.001 unit*	Dog	Ejection	Vasoconstriction	Linzell, 1950
	0.01 " *	Cat	"	"	" "
	0.5 "	Sow	"	-	Whittleston 1952
	1-10 units	"	"	-	Braude & Mitchell, 1950
Pitressin (vasopressor hormone)	10 units	Cow	Partial ejection	40-60% decr. in blood flow	Petersen, 1942
	0.5 unit	Sow	" "	-	Whittleston 1950
	0.01 milli-unit	Dog	No ejection	Vasoconstriction	Linzell, 1950
	0.01 unit	Rabbit	Ejection	-	Cooper & Turner 1941
Adrenaline	50-200 ug	Cow	Partial ejection	50-100% decr. in blood flow	Petersen 1942
	50-200 ug	"	Little or no ejection	-do-	Peeters et al 1952
	0.0001 ug*	Dog	-	Vasoconstriction	Linzell 1950
	0.005 ug*	Cat	-	"	" "
	10^{-6} ug*	Dog	-	"	Linzell & Hebb 1951
	10^{-7} ug*	Cat	-	"	" " "
	10^{-4} ug*	Goat	-	"	" " "
	0.2 ug	Sow	inhibits ejection	-	Whittleston 1952

Table I (Contd.)

Substance	Amount	Species	Milk ejection response	Vascular response	Reference
Acetyl-choline	4-100 mg	Cow	Complete ejection	No effect	Petersen, 1942
	20-100 mg	"	Partial "	Increased blood flow	Peeters et al 1952
	0.1 ug	Dog	-	Vasodilatation	Linzell, 1950
	0.2 ug*	Cat	-	"	" "
	0.2 g	Sow	Partial ejection	-	Whittleston, 1952
Histamine	0.5-10 mg	Cow	Partial ejection	10-50% decr.in blood flow	Petersen, 1942
	5-100 mg	Cow	" "	Decreased blood flow & capillary dilatation	Peeters et al, 1952
	0.1 ug	Cat & Dog	-	Vasodilatation	Linzell, 1950
	0.4 mg	Sow	No effect	-	Whittleston, 1952
Atropine	2-4 mg	Cow	- prevented	Slight incr.in blood	Petersen, 1942
	2-4 mg	"	- action of	- flow	Peeters et al, 1952
	50 ug	Cat & Dog	- acetylcholine	Varied	Linzell, 1950
Ergonovine	0.2-0.6 mg	Cow	- prevented	10-20% decrease in	
Di-hydro-ergotamine	100 ug	Cat & Dog	- action of adrenaline	blood flow varied	Petersen, 1942 Linzell, 1950
Dibenamine	7 mg/kg	Cow	-	-	Peeters et al, 1952
Neoantergan	40 mg	Cow	Partial ejection	Prevented action of histamine	Peeters et al, 1952
Carbamyl-choline	2-8 mg	Cow	No effect	Slight increase	Petersen, 1942

* - Minimal effective doses. - = No observation available

St.Clair (1940) presented a thesis to the Iowa State College entitled "The Nerve Supply of the Bovine Mammary Gland." His work led him to the conclusion that the bovine udder was innervated by sensory and sympathetic fibres only, these reaching the udder by way of the inguinal nerve, the first two lumbar ventral branches and the perineal nerves. He was unable to find gauglia in the udder, and as a result, he believed that the mammary gland did not have a parasympathetic supply. By cutting the inguinal nerve he caused a vasodilation of the udder, with a resulting increase of local temperature, indicating that the sympathetics were constrictors to the peripheral blood vessels. St.Clair supposed that the sympathetic fibres had a stimulatory effect on smooth muscle elements of the udder, and even though a hormonal influence overshadowed any nervous action the influence of the sympathetic system could not be denied.

Petersen (1942) carried out pharmacological studies on the mammary gland. These led him to believe that the bovine udder was innervated by both a sympathetic and parasympathetic supply, for he argued that a response to acetylcholine and adrenalin indicated a cholinergic i.e. a parasympathetic, and a sympathetic supply.

Peeters, Coussens and Sierens (1949) using a

perfused gland, studied the effect of inguinal nerve stimulation in the presence of ganglionic blocking agents, for parasympathetic innervation is always associated with the presence of nerve gauglia in the tissue supplied. Electrical stimulation of the inguinal nerve led to vasoconstriction, a decrease in milk pressure and the onset of teat contractions, such effects being unaltered by the ganglionic blocking agents employed (nicotine and tetraethyl ammonia). Similar effects to those of nerve stimulation were produced by the injection of adrenalin, and were likewise inhibited by dibenamine. These workers interpreted their results as indicating the absence of a parasympathetic supply. In a further paper Peeters, Genie and Coussens (1951) examined the gland for the release of acetycholine during nerve stimulation using an eserinised perfusion fluid (eserin being a compound that inhibits the activity of choline esterase). They were unable to detect any increase in the activity of leech muscle to this fluid after stimulation. - a result indicating the absence of parasympathetic fibres in the udder, for leech muscle is particularly sensitive to the presence of acetycholine.

Linzell (1950) made a detailed study of the vasomotor nerve supply to the mammary gland of the cat and the dog. He reported vasoconstriction in response to electrical stimulation of the external spermatic and mixed spinal nerves by observing decreased venous

outflow from the isolated gland, increased perfusion pressure, and microscopical examination of living blood vessels. Ergot preparations abolished the vasoconstrictor response, adrenergic stimulants potentiated it. Intra arterial administration of adrenalin completely simulated the effects of nerve stimulation. Although the mammary blood vessels responded to acetylcholine by vasodilation, this observation was not regarded as evidence for cholinergic innervation since the effects of nerve stimulation were unaffected by eserine, atropine or nicotine - all ganglionic blocking agents.

In view of this evidence it may be concluded with considerable confidence that the udder has a vasomotor nerve supply of a sympathetic nature, a supply of which probably exerts its vasoconstrictor influence by means of the secretion of an adrenalin like substance. The evidence available indicates the absence of a para-sympathetic supply in the udder.

The Physiology of the Interaction of Pitocin and Adrenalin.

The mechanism whereby adrenalin inhibits the action of Pitocin is an interesting but yet undecided problem. Hebb and Linzell (1951) showed that the sensitivity of mammary blood vessels to adrenalin is dependent upon the rate of blood flow through the gland. Because of the sensitivity of the mammary

blood vessels to adrenalin, it has been postulated that the inhibiting action of adrenalin on the response to pitocin in the mammary gland, reported by many workers (Peeters et al, 1949, 1952, Whittleston 1951,), is due to the vasoconstriction caused by adrenalin preventing the access of pitocin to the contractile elements of the gland.

Observation of the behaviour of smooth muscle "in vitro" in response to adrenalin indicates that adrenalin may act directly upon the smooth muscle causing a marked relaxation (Chapter 8). In view of the fact that myoepithelium and smooth muscle are capable of exhibiting a similar staining reaction (Linzell 1951), and behave in a parallel manner to the action of pitocin, it is not an unwarrantable assumption that adrenalin might well cause a relaxation of myoepithelium similar to that caused in smooth muscle.

If we make the assumption that the action of these drugs is at the surface of the cell, it is feasible to visualize a mechanism whereby a concentration of the molecules concerned, sufficient to stimulate contraction, might arise at a cell surface that exhibits a high degree of specificity.

Adrenalin contains ionizing groups, polar groups and non-polar hydrocarbon groups in its molecule, hence it has an intrinsic capability of

being adsorbed at a surface, and further, when adsorbed, of altering the properties of the surface concerned.

Danielli (1950) furnishes an excellent example of the problem under consideration.

Consider the concentration of adrenalin which may arise at a surface. This is given by Boltzmann's theorem :

$$\frac{Cs}{Cb} = e^{\frac{E}{RT}}$$

where Cs = concentration at adsorbing surface

Cb = bulk concentration

E = energy of adsorption

R = gas constant

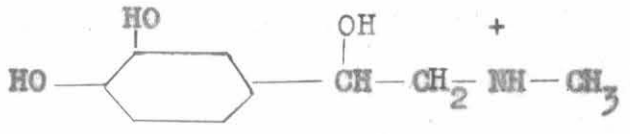
T = absolute temperature

e = the expotential function.

The energy E may be regarded as made up of 3 components, one associated with ionic groups, one with the polar groups, and one with the non-polar groups.

For adrenalin the minimal values of these are -

Ionic	Polar	Non-polar	Total E	$\frac{Cs}{Cb}$
700	3000	3500	7200	2×10^5

Adrenalin 

Thus the concentration of adrenalin which may arise at a surface is seen to be of the order of 10^5 times that which is found in the bulk phase

i.e. the circulating blood.

The surface at which a drug is adsorbed must present an organisation of ionizing groups, polar groups and non-polar groups as specific as that which is to be found in the drug itself. If this criterion is filled the possible energy of adsorption is large - but a group on the wrong position or having the wrong orientation may readily prevent the dove-tailing of the drug and the surface, thus preventing many of the sites of potential adsorption becoming effective.

Considering the mechanism of both adrenalin and pitocin pharmacology in this manner, it is feasible to imagine that the antagonism between them might take the form of adrenalin disrupting the adsorbing surface of the myoepithelial cell preventing the surface uptake of the oxytocic factor, for it is probable that pitocin acts on the muscle cell by this same selective surface adsorption phenomena.

This hypothesis becomes clearer when we consider the functions of enzyme in a muscle cell, for these include the synthesis of substances acting as an immediate source of potential energy, e.g. the synthesis of adenosine triphosphate, and the conversion of this potential energy to mechanical

work, as is seen in muscular contraction.

The possible mode of activity of pitocin in any enzyme system must be purely speculatory, yet it is convenient to think in terms of its action as a prosthetic group of an enzyme, or as a co-enzyme, whereby it rapidly activates a complex metabolic reaction.

S U M M A R Y

The development of the neuro-endocrine theory of milk let-down is reviewed in some detail. Alternative explanations of the phenomenon of active milk ejection are discussed and rejected in favour of this theory.

The role of the autonomic nervous system in the ejection of milk from the mammary gland is discussed. It is concluded that only sympathetic fibres are present in the glandular tissue, these having a vasoconstrictor action on the mammary blood supply.

The influence of adrenalin on the mammary gland is compared with the vasoconstrictor sympathetic effect, and the mechanism of an alternative theory to that of vasoconstriction explaining the inhibiting effect of adrenalin on milk ejection is postulated.

The mode of action of Pitocin on myoepithelial cells is suggested.

THE POSTERIOR PITUITARY AND ITS ROLE IN MILK EJECTION

The evidence available concerning the nature of the neuro-endocrine mechanism involved in the let-down of milk, indicates that the posterior lobe of the pituitary is the essential endocrine gland involved. The mechanism of transmission of the nervous impulse in afferent and efferent sensory and motor neurones, such as those linking the mammary gland, the central nervous system and the posterior pituitary, is well reviewed in many texts, consequently it is not proposed to discuss the problem here. However a knowledge of the physiology of the posterior pituitary lacks such ready accessibility. Hence it is considered advantageous to this study to review the relevant literature, placing special emphasis on the innervation of the gland, and its products of metabolism.

The Anatomy of the Posterior Pituitary

The pituitary gland originates as a result of the conjugation of an epithelial upgrowth from the stomodaeal region (the hypophysis) with a downgrowth from the third ventricle of the brain (the infundibulum). In the adult animal the hypophysis is recognizable as the pars tuberalis and glandularis, forming the anterior pituitary, and

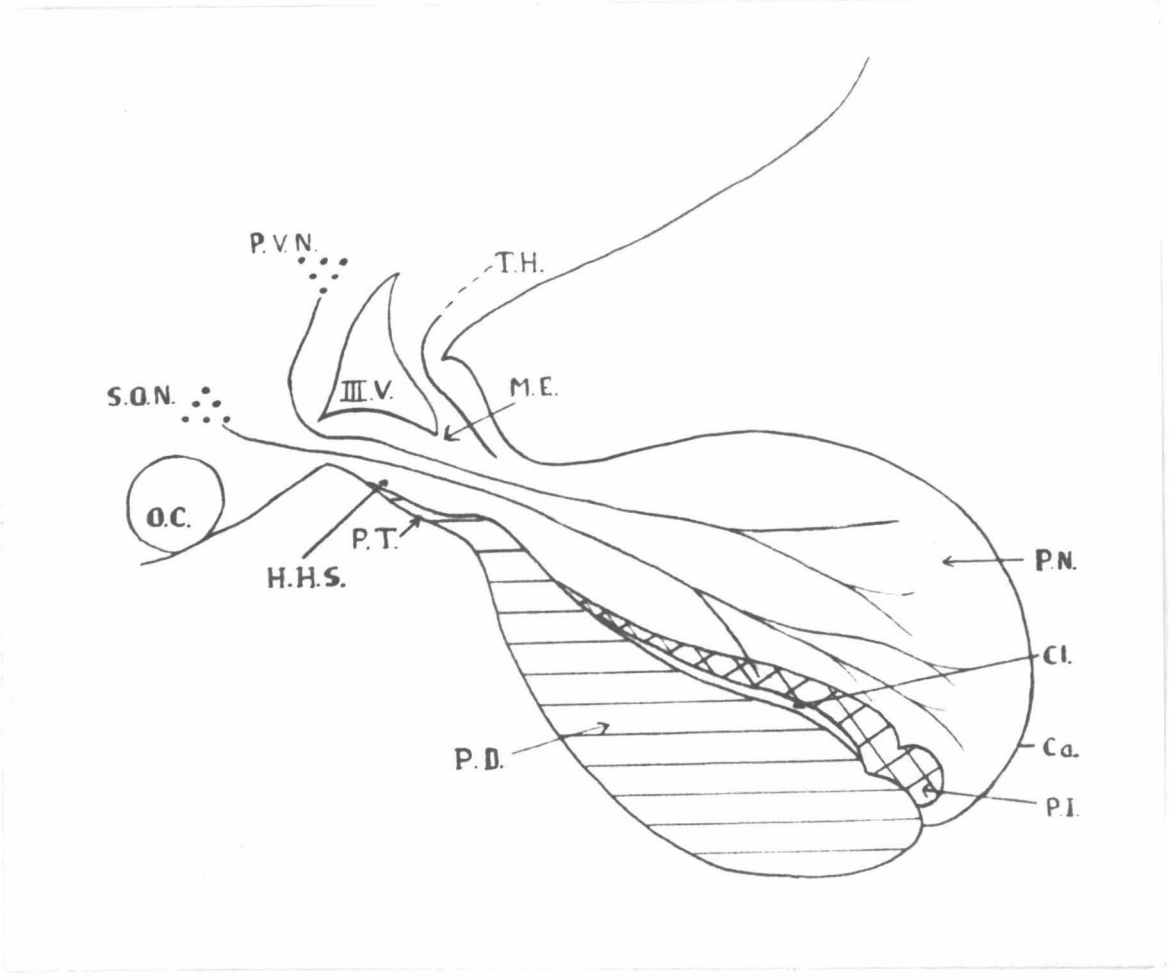


Fig. 2.1 - Diagramatic representation of a median longitudinal section through the neuro-hypophysis. (P.V.N. - Para-ventricular nucleus, S.O.N. - Supraoptic nucleus, O.C. - Optic chiasma, H.H.S.-Hypothalamo hypophyseal stalk, P.T. - Pars tuberalis, P.D. - Pars distilis, P.I. - Pars intermedia, Ca - Capsule, P.N. - Pars nervosa, M.E. - Median eminence, IIIv - Third ventricle, T.H. Other fibres of the tubero - hypophyseal tract.)

the pars intermedia forming the intermediate lobe, both being of epithelial origin, while the infundibulum gives rise to the posterior pituitary, this being of nervous tissue origin. The division of the pituitary body of most mammals at the hypophyseal cleft results in the separation of the gland into an anterior and posterior lobe, the latter containing both neural and epithelial tissue and more correctly designated as the neuro-intermediate lobe. The term neurohypophysis is usually confined to the neural tissue of the posterior pituitary, i.e. the neural lobe or infundibular process, the neural stalk or infundibular stem, and the median eminence of the tuber cinerium, the three portions apparently functioning as a unit. (Nomenclature from Rioch, Wislocki and O'Leary 1940).

The Pharmacological Activity of Extracts of the Posterior Pituitary

About the turn of this century physiologists commenced the study of the pharmacological activity of extracts of the posterior pituitary. It was found that the injection of such extracts had a pressor action (Oliver and Schafer 1895), a diuretic action in anaesthetized animals (Magnus and Schaffer 1901), an antidiuretic and chloruretic action in unanaesthetized animals (von den Velden 1913) a hyperglycaemic action (Borchardt 1908), a stimulating action on intestinal

peristalsis (Bell 1909), an oxytocic action (Dale 1909) and a galactogogue action (Ott and Scott 1910). Kamm et al in 1928 purified this single extract with multiple activities, and separated it into two fractions. The names and probable activities of these two fractions are shown below :

		Probable activities
Whole posterior pituitary extract ("Pituitrin" Parke Davis & Co.)	Pressor fraction ("Pitressin" Parke Davis & Co.)	pressor antidiuretic chloruretic stimulation of intestinal peristalsis hyperglycaemic
	Oxytocic fraction ("Pitocin" Parke Davis & Co.)	oxytocic galactogogue

Oral administration of the dried gland, or an extract of the gland is without obvious physiological action; however intravenous subcutaneous or intramuscular injections of aqueous extracts produce marked effects on many organs. This effect is modified by such factors as anaesthesia, mode of administration, state of estrus, stage of pregnancy, in vitro or in vivo observation, and the introduction of substances into the extract modifying its active constituents or delaying their adsorption.

Waring and Landgrebe (1950) ably reviewed the literature pertaining to the pharmacology of the gland extracts. A summary of their review is presented in Table II.

T A B L E - II

PHARMACOLOGY OF NEURAL LOBE EXTRACTS

Response	Effects Exhibited after Injection of Ox Extract						
	Unanaesthetized animals		Anaesthetized animals		Organ In Vitro	Activity in	
	I.V.*	Other ø	I.V.*	Other ø		Pressor fraction	Oxytocic fraction
1. Contraction of uterus	+	+	+		+	?	+
2. Inhibition of water diuresis	+	+			+	+	?
3. Inhibition of tubular resorption	+	+				?	+
4. Potentiation of kidney diuresis		+	+			?	?
5. Elevation of blood pressure			+	+		+	-
6. Depression of blood pressure	+		+			?	?
7. Coronary constriction	+		+		+	+	-
8. Dilation of hepatic veins					+	-	+
9. Inhibition of coronary constriction						-	+
10. Dilation of coronary vessels					+	-	+
11. Contraction of capillaries	+	+	+	+	+	+	
12. Inhibition of secretion of gastric juice, particularly HCl							
13. Hyperglycemia	+	+	+			+	?
14. Stimulation of intestinal muscles	+	+	+		+	+	+
15. Galactogenic action	+	+	+		+	+	+
16. Facilitation of sleep		?					
17. Effect in fat concentration		+				+	-
18. Reduction of lymph + oedema fluid production	+	+					

* - Intra Venous ø - Other Injection Routes

The Site of Formation of the Active Constituents

Owing to the fact that neurohypophysis bears little resemblance to a glandular structure, controversy has arisen as to the site of origin of the pharmocologically active substances found in the gland.

The pars nervos and pars intermedia of the posterior pituitary are closely adherent structures, and are not separated in making the usual posterior lobe extracts used in experimental and clinical work. In an endeavour to distinguish the role of each tissue, Van Dyke (1926) made a mechanical separation of the pars intermedia and pars nervosa, controlling the experiment by microscopical examination of the separated tissues. Extracts of the pars nervosa, free from the pars intermedia, contained many times the oxytocic pressor and antidiuretic activity present in extracts of the pars intermedia. On the other hand, extracts of the pars nervosa had no action in expanding the melanophores of the frog, this property residing only in the pars intermedia. This work was confirmed by De Lawder, Tarr and Geiling (1934) who demonstrated the presence of oxytocic, pressor, and antidiuretic activities in the pars nervosa of the fowls pituitary, a tissue free of pars intermedia adherence. These workers were of the opinion that these three activities were

associated with substances formed by the endogenous constituents of the nervosa.

There are many histological features in common between the pars nervosa, the pituitary stalk, and the median eminence of the hypothalamus - a not unusual fact in view of their identical embryological origin (Fisher et al 1938). Similarity of blood and nerve supply to the three tissues has led Fisher et al to postulate that these structures are part of the one secreting tissue, the neurohypophysis.

The evidence upon which the actual site of origin of these pharmacological substances might be determined, may be resolved into five groups :

1. The distribution of colloid and humeral activity after stalk section.
2. Tissue cultures of the neural lobe.
3. Comparative assays of the component tissues of the posterior pituitary.
4. Electrical stimulation of various portions of the neurohypophysis and hypothalamic area.
5. Studies on neuro-hypophysectomized animals.

Fisher et al (1938) and O'Connor (1947) reported that sectioning of the pituitary stalk resulted in a loss of posterior pituitary activity. Ranson (1936) produced electrolytic lesions in the hypothalamus causing a permanent diabetes insipidus

in the experimental animals, a condition indicative of posterior lobe dysfunction. Bargmann and Scharrer (1951) reported that the cutting of the stalk resulted in an accumulation of colloid material in the pituitary tract, this colloidal material being formed in the neuro secretory cells of the hypothalamic area and moving via the axoplasm of the tractus preoptico hypophyseus to the pars nervosa or the point of section, and accumulating there (Fig.2.2). They identified this colloid with the active principles of the pars nervosa, for they reported characteristic posterior pituitary activity in the hypothalamus , and correlated the amount of this colloid with the state of hydration in rats. Their conclusion was that the pars nervosa merely stored, but did not produce the stainable material, i.e. the active principles, which it contained.

The identification of gland cells amongst the fibres of the pars nervosa has been reported by a number of workers, e.g. Bucy (1930), Griffith (1938), while they have been observed in sections prepared in this laboratory (Fig.2.3). Geiling and Lewis (1935) made tissue cultures of the pars nervosa of the mouse, and in so doing inevitably separated each nerve fibre from its perikaryon in the supra optic nucleus, thus precipitating Wallerian degeneration. A pressor response was recorded 50 days after ex-

plantation, the only characteristic elements present being extensive networks of large cells resembling neuroglia cells. No test for oxytocic or anti-diuretic response was made.

Whittleston (1951) has carried out numerous assays of the milk ejecting activity of the posterior lobe, the stalk and the median eminence. Invariably he found far greater activity in the posterior lobe, with traces of activity in the median eminence and the stalk, activity which he attributed to an incomplete dissection of the vascular bed upon which the gland lay. These results are in agreement with the comparative assays of Van Dyke (1926) and Sato (1928).

Cross and Harris (1950) succeeded in placing a glass covered platinum electrode, mounted in a stereotaxic instrument, in the pituitary stalk of a rabbit, and by applying a stimulation of 1.0 V at 50 cycles A.C. per second, recorded a milk ejection response after 25 seconds rising to a peak at 90 seconds and subsiding within 4 to 7 minutes. Previously Harris (1948) and Koella (1949) had shown that a similar type of electrical stimulation of the anterior hypophalamus and pituitary stalk produced an increased secretion of the antidiuretic substance from the area. Andersson (1951) using lactating sheep and goats was able to demonstrate

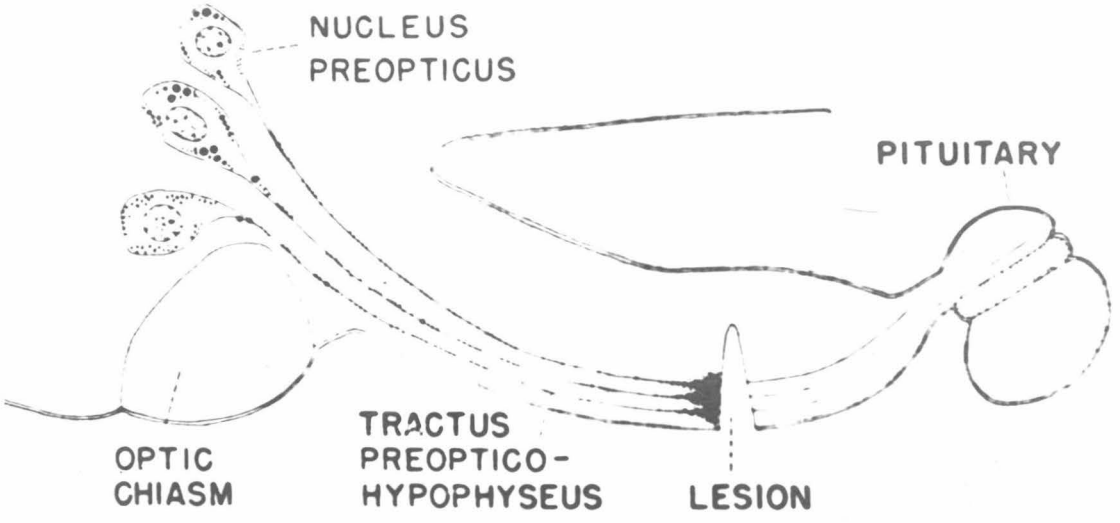


Fig.2.2 - Diagramatic representation of the accumulation of colloid in a sectioned hypothalamico hypophyseal tract. (From Bargmann and Scharrer)

milk ejection in sacral anaesthetized animals, or denervated animals, when the hypothalamus was subject to electrical stimulation, the region of the supraoptic nuclei being the only area capable of responsive stimulation. Associated with a milk ejection response was one of antidiuresis; a confirmation of the observations of Harris (1948) and Koella (1949). Chang Lim et al (1937) had previously demonstrated a vago pituitary pressor reflex by stimulating the vagus, while Huang (1938) produced evidence suggesting connections between the vagus and supraoptic nuclei, thus providing a mechanism for the reflex observed by Chang Lim and associates. Andersson (1951) repeated the stimulation of the vagus, obtaining a milk ejection response in both normal and denervated animals.

The induction of diabetes insipidus by the extirpation of the pituitary, or by stalk section, has been reported by many workers. The effect of pituitary extirpation on parturition is however controversial. Smith (1932) and Allan and Wiles (1932) using rats and cats respectively, reported a normal parturition after hypophysectomy. On the other hand, Fisher, Ingram and Ranson (1938) observed that cats with an atrophy of the pars nervosa following section of the supraoptico hypophysial tracts, had normal pregnancies, but at parturition

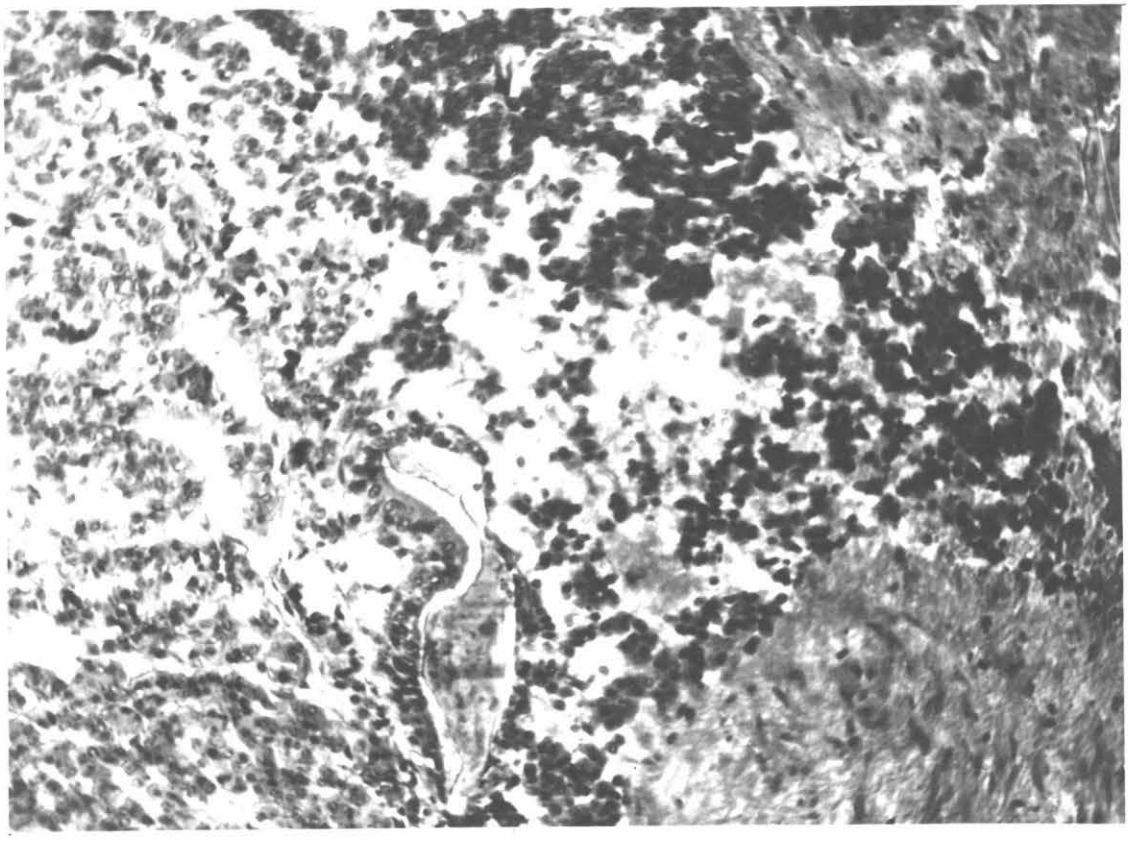


Fig.2.3a - A general low power view of the three tissue constituents of the pituitary gland (x 300) Mallory.

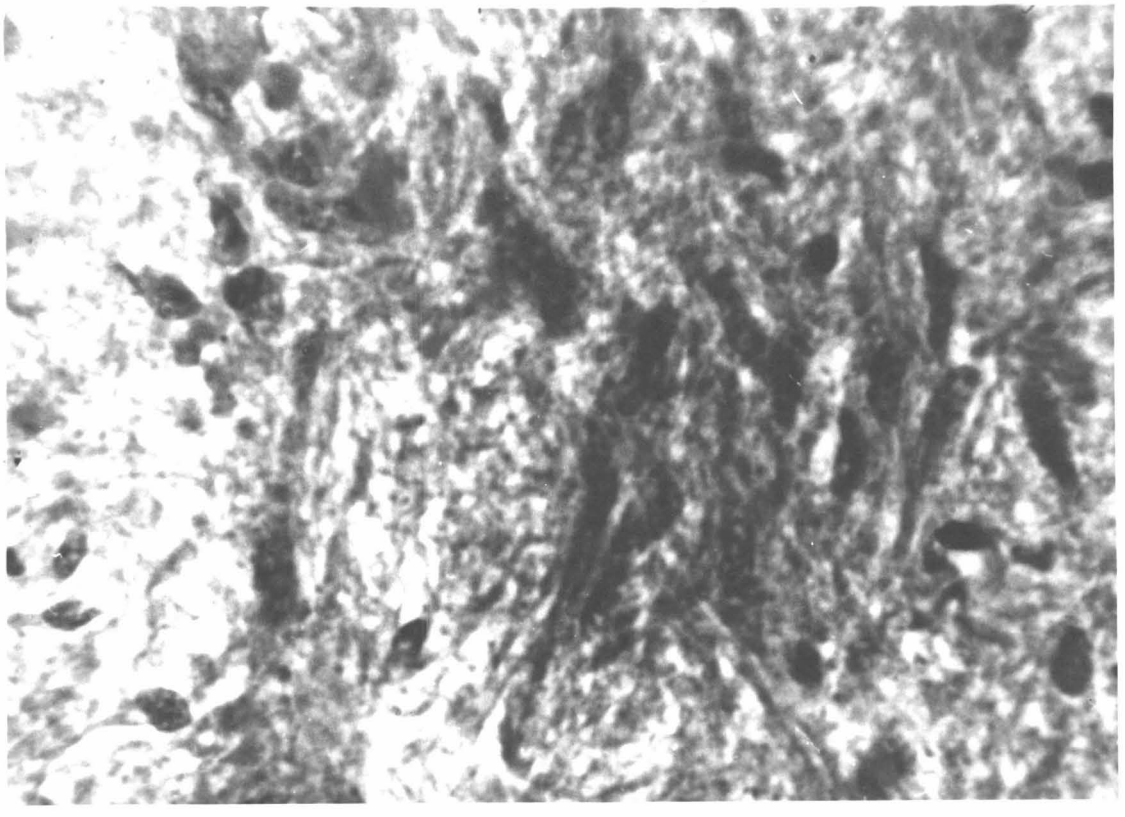


Fig.2.3b - A high power view of the Pars nervosa (x 900). Imposed on a background of nervous tissue may be seen cells with a granulated cytoplasm, these structures being the possible source of the active constituents of the gland. Mallory.

the uterine contractions were feeble and the animals died with the foetuses undelivered. The explanation probably takes the form that the posterior pituitary is the normal mechanism involved in parturition. In its absence alternative uterine contracting mechanisms take over its function.

For the moment no conclusive decision as to the source of the posterior pituitary factors may be propounded. However it seems likely that these factors originate in the posterior lobe, and that the function of the nerve fibres is to supply a reflex connection between the mammary gland via the supraoptico hypophysial tract, to the neural lobe.

The Nerve Supply of the Neurohypophysis

The posterior pituitary provides a paradox in the study of endocrinology, for it would appear that whilst most endocrine glands either lack a nerve supply or receive only a scanty innervation, the posterior pituitary, along with the adrenal medulla possesses an abundant innervation.

In an endeavour to further elucidate the general neuro-endocrine relationship, Harris of Cambridge University, has made an intense study of the innervation of the gland. It is largely from his publications that the following notes are drawn.

The nerve supply of the neurohypophysis is

derived from two sources, a scanty sympathetic supply from the carotid plexus running with the posterior median hypophyseal artery into the posterior lobe of the gland, and a hypothalmic supply which reaches the infundibular lobe through the neural stalk. Little is known regarding the function, cells of origin or termination of the sympathetic supply. The hypothalmic supply has however been the subject of much detailed study, which is reviewed by Harris (1943) (Fig.2.1).

The hypothalmico-hypophysial tract has been divided into the supraoptico hypophysial tract running in the ventral wall of the neural stalk, and the tubero hypophysial tract in the dorsal portion of this structure. It appears that the fibres of the hypothalmico-hypophysial tract originate in the supraoptic and paraventricular cells of the hypothalmus and terminate mainly in the neurohypophysis, a few penetrating the pars tuberalis, pars intermedia and possibly the pars distalis.

The Neural Control of the Neurohypophysis.

Information regarding the functional relationship between the nervous system and the neurohypophysis has been arrived at in three main ways. Studies have been made of the effects of (a) lesions of the supraoptico hypophysial tract, (b) changing the internal environment in such a way that the secretion

of the gland is increased, and (c) direct electrical stimulation of the nerve supply of the gland.

(a) Lesions of the Supraoptico hypophysial tract.

Fisher, Ingram and Ranson (1938) were able to place localized lesions in different parts of the hypotholamus of cats. They noted that lesions which bilaterally interrupted the supraoptico-hypophysial tract resulted in a condition similar to that of clinical diabetes insipidus, involving a polyuria that could be returned to normal only by replacement therapy with posterior pituitary extracts. Post mortem examination of the pituitary region revealed that atrophy of the neurohypophysis had occurred; the median eminence and the infundibular stem and process were shrunken and hypercellular, although the pars intermedia and pars distalis appeared normal. The occurrence of nerve fibres in the neurohypophysis in cases of marked diabetes insipidus was almost negligible , while extracts of these atrophic glands were practically inactive in terms of pressor oxytocic and antidiuretic response. These results were later confirmed by similar experiments on the monkey (Fisher et al 1938).

The data regarding secretion of the oxytocic substance by the neurohypophysis following interruption of the supraoptico hypophysial tracts

is not so complete as that regarding the antidiuretic hormone. Harris (1951) reviewed the problem and summarized the situation by stating that a large proportion of animals in which the supraoptico hypophysial tracts had been divided, had a prolonged labour, or delivered their young dead.

Cross and Harris (1952) and Andersson (1951) have independently shown that lesions in the supra-optico hypophysial tract in lactating rabbits, and sheep and goats respectively, caused a marked diminution in the quantity of milk obtained in standard suckling tests, and incomplete evacuation of the mammary gland. Replacement therapy with posterior pituitary extract increased the amount of milk available to the young and evacuated the mammary gland.

(b) Indirect Stimulation.

The information available is confined to antidiuretic responses and was reviewed by Verney (1947 - 1948). Verney concluded that emotional stress resulted in excitation of the supraoptic nuclei leading to an increased rate of secretion of the antidiuretic hormone, while changes in the osmotic pressure of the arterial blood led to a posterior pituitary response in terms of the secretion of antidiuretic hormone, restoring either the hypo or hyper tonic osmotic pressure to normal.

(c) Direct Electrical Stimulation of the Supraoptico hypophysial Tract.

Early attempts at stimulating the supraoptico

hypophysial tract were frustrated by complications introduced by anaesthesia (Haterius and Ferguson 1938), for anaesthesia itself affects the rate of urine secretion, the level of blood sugar etc. Harris (1948a) overcame this problem by the use of a remote control technique enabling him to stimulate the basal regions of the brain without anaesthesia. In a preliminary operation a small coil was buried between the scalp and the skull, and an insulated electrode soldered to one end of this coil, carried down through the skull, corpus collosum, and other midline structures into some part of the hypothalamus or pituitary gland. After recovery from the operation the tissue surrounding the electrode tip could be stimulated by holding a primary coil carrying an A.C. current, over the animal's head so that the embedded secondary coil was situated in an electromagnetic field.

Stimulation of the supraoptico hypophysial tract by this method has been shown to result in the liberation of the antidiuretic hormone and an oxytocic substance.

Cross and Harris (1952) and Andersson (1951) working independently confirmed the milk ejection response in lactating rabbits and in sheep and goats. Both workers attributed milk ejection to the liberation of a hormonal factor.

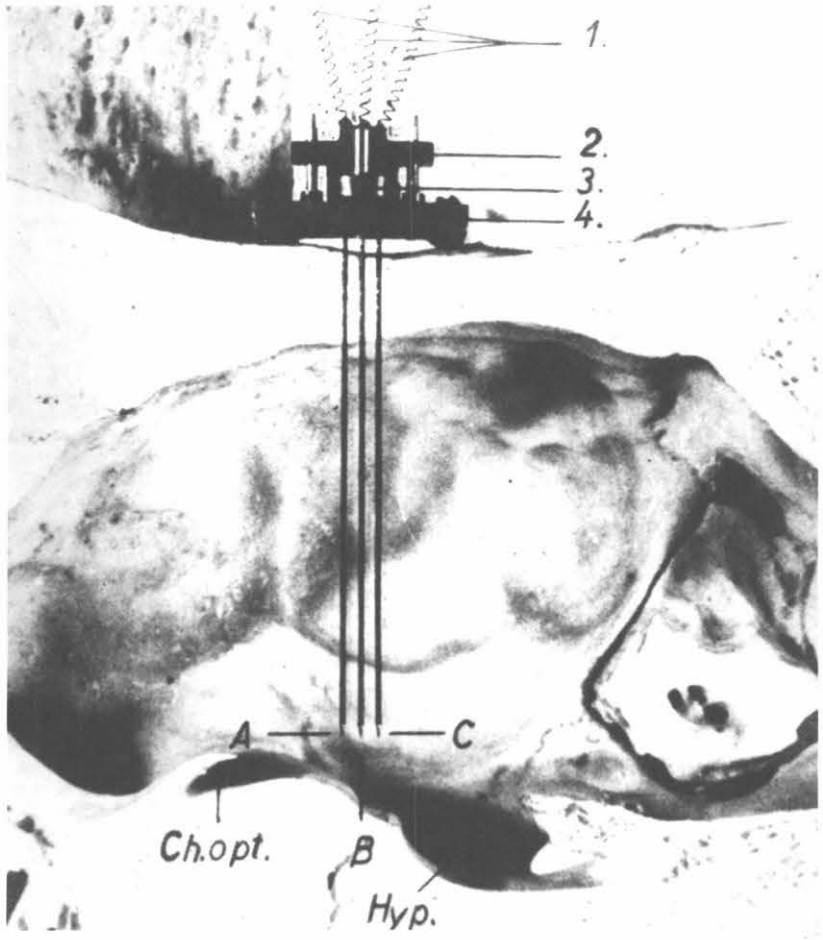


Fig.2.4 - An X-ray picture of the electrodes of a stereotaxic instrument placed in the hypothalamus of a goat. El - Electrodes, Ch.opt. - Chiasma opticum, Hyp. - Position of the pituitary gland. (From Andersson)

The essentiality of the posterior pituitary to lactation, suggested by Gomez in 1939 was confirmed by Cross and Harris (1951, 1952) for they found that lactating rabbits with lesions of the supraoptico hypophysial tract, or with this tract severed, were unable to successfully suckle their young. The use of posterior pituitary replacement therapy overcame the problem.

The Nature of the Substance Liberated from the Neurohypophysis.

Early views attributed the pharmacological activities of the posterior lobe of the pituitary gland to adrenalin and later to histamine, a theory disproved by Dudley in 1919. The numerous phyarmacological effects of neural lobe extracts led at an early stage to two schools of thought, one believing in the existence of a single hormone with multiple activities, the other attributing a separate substance to each main activity. Kamm et al (1928) reviewed these early ideas.

Two closely related problems are involved. First the nature of the substance or substances elaborated and stored by the gland, and secondly the nature of the substance(s) liberated from the gland into the blood stream.

Van Dyke et al (1942) isolated from the dried posterior lobe, a protein which behaved as a

homogeneous substance to solubility, electrophoresis and ultracentrifugation tests, and which possessed oxytocic pressor, and antidiuretic activities in the same proportion as those of the U.S.P. reference standard. These authors suggested that even if the pars nervosa elaborated a single protein with multiple activities, it was possible that specific enzymes liberated active fragments of the parent molecule into the blood stream, depending on the requirements of the organism. As is well known, posterior pituitary extracts may be fractionated into preparations showing a high degree of separation of pressor and oxytocic activities (Kamm 1928), a fact difficult to reconcile with Van Dyke's views. The pressor principle is usually considered responsible for the renal action of extracts, however according to Heller (1939) some separation of these activities is possible - but this work in turn was questioned by Fraser (1942) who maintained that the hydrolysis as used by Heller involved no real change in the ratio of antidiuretic and pressor activity.

The chemistry of the posterior pituitary factor(s) was well reviewed by Whittleston (1948) and by Waring and Landgrebe (1950). Whittleston summarized his review in a concise table reproduced in Table III.

TABLE - III

For the Unitarian Theory	Against the Unitarian Theory
1. All activities are simultaneously destroyed by alkali.	1. Oxytocic activity may be separated from pressor and antidiuretic activity by chemical means.
2. Tryptic digestion and acid hydrolysis give parallel inactivation.	2. The two hormones differ in amino acid content.
3. Pressor, oxytocic, and antidiuretic activities diffuse at the same rate.	3. The pressor constituent of simple gland press juice moves more quickly in an electric field than does the oxytocic hormone.
4. All activities behave in the centrifuge as if associated with a single protein molecule.	4. Adsorption on artificial zeolite readily separates the activities.
5. An apparently pure protein with oxytocic pressor and antidiuretic activities can be isolated from the pars nervosa.	

Waring and Landgrebe (1950) carried out oxytocic and pressor assays of different glands and though their work was limited, the pressor oxytocic ratio was constantly about the mean of 1:1, suggesting a common identity. Further, they argued that if the gland initially manufactured more than one excitant, it should be feasible to withdraw one, leaving the other(s) "in situ". Several methods of stimulating posterior pituitary secretion are known. Of these they used the injection of hypertoxic sodium chloride, dehydration and vagel stimulation. After the appropriate treatment the glands were assayed separately for oxytocic and pressor activities. All procedures caused a reduction of residual activity of the gland,

and in all cases the ratio of pressor to oxytocic activity was the same as in the controls.

Yet to contradict this a number of workers have observed that the substance(s) liberated from the neurohypophysis on stimulation of its nerve supply exerted relatively less pressor or anti-diuretic activity as compared with oxytocic than did whole posterior lobe extracts (Ferguson 1941 Harris 1947, 1948).

The fact that pitressin is capable of eliciting a milk ejection response of its own accord (Turner and Cooper 1941, Andersson 1951, Whittleston 1952) illustrates what is probably a multiple function of this "hormone", and thereby queries the necessity of a dual hormone hypothesis.

A final decision will depend on the isolation of an undoubtedly pure substance with multiple activities, on a weight basis consistent with the quantities known to be stored in the gland. Until this is achieved the unitarian theory must remain an interesting hypothesis.

S U M M A R Y

The anatomy of the pituitary gland is briefly described.

Extracts of the posterior pituitary exhibit a marked pharmacological action on the mammalian body when administered by any means other than an oral route. Four major activities may be ascribed to the unfractionated extract - a pressor action on the blood vascular system, an antidiuretic activity, an oxytocic action and a galactogic effect. In fractionated extracts the pressor and antidiuretic activities appear associated, likewise the latter two.

The evidence available favours the view that the site of formation of these active substances is the posterior lobe of the pituitary gland.

The neurohypophysis is richly innervated by the supraoptico hypophysial tract of nerve fibres. If this tract is interrupted secretory activity of the gland ceases, the gland atrophies, diabetes insipidus, dystocia, and failure to let-down milk follow. It is concluded that the neurohypophysis is essential to normal lactation and is dependant upon nervous connections with the hypothalamus for its continued function.

Strong evidence exists showing that this tissue produces a hormone as yet specifically unidentified involved in the normal milk ejection mechanism. Other active substances of unknown relationship to the milk ejecting hormone are also periodically liberated from this tissue.

PART - II

EXPERIMENTAL

THE QUANTITATIVE MEASUREMENT OF MILK EJECTION IN THE SOW

A Description of the Technique Used

The technique of Whittleston (1952) has been utilized in an endeavour to derive an accurate assay procedure for the milk ejection hormone, and to make a study of factors affecting the let-down response. (See Introduction)

Administration of pitocin to a lactating animal involves the use of an intravenous injection, for intramuscular and subcutaneous injections do not provide a rapid quantitative response of milk ejection. Intravenous injection in the sow is feasible only by using the ear vein, for while it is possible to enter the anterior vena cava (Carle and Dewhurst 1942), and possibly the radial and caudal veins, the restraint necessary renders multi-injection schedules impractical. Accordingly a crush bale was built allowing easy entry and exit for the sow, body movement being restricted however. In this bale the sow's ears were readily examinable, and with practice, intravenous injections readily made. Preliminary work indicated that the number of vein punctures made in any one sow over a restricted period of time would be definitely limited, for haematomas formed readily, while the tissue about the vein enlarged considerably after injection.

This limitation was a distinct disadvantage to experimental work. Cannulation of the ear vein was the obvious answer. It was found that this could be accomplished rapidly and simply by the insertion of a 22 gauge 1-1/4 inch Luer needle into the vein, the needle being held in position with adhesive tape.

The syringe used was a tuberculin type B.D. Yale make, calibrated with a double scale of minims and cubic centimetres, the latter reading to 1/100th c.c. With a capacity of 1 c.c. the syringe was capable of delivering this amount with an accuracy of one part in 200, as indicated by delivery weight calibration. The simple needle syringe union facilitated the cannulation procedure, and provided the sow was tolerably quiet, up to 20 injections were readily given.

Pitocin was used as the source of the milk ejection hormone. This is a Parke Davis preparation of the oxytocic fraction of the posterior lobe hormone(s). It had an activity of 10 units per c.c. and a guarranteed pressor contamination of not more than 5%. For injection purposes this preparation was diluted in the ratio, 1 part pitocin: 9 parts saline, giving a solution with a potency of one unit per c.c. A half unit dose i.e. $\frac{1}{2}$ c.c. was thus a convenient volume with which to work.

In the summer of 1951 when this work was commenced approximately 13 sows were available for use. These consisted of animals of three breeds, Berkshires, Tamworths, and Large Whites. In itself this was both an advantage and a disadvantage. The diversity of breed type of the sows might logically be expected to contribute to greater variability of experimental results, and hence confound precise conclusions. However when investigating the properties of a substance in a new field of work, it may be undesirable to confine ones experiments to a very homogeneous population of animals, as misleading results may be obtained, which however true for the particular stock and test conditions utilised, may not be of wide application to other even closely related biological material. Regard has therefore been paid to the distinction between the frequent desirability of rigid control of conditions in a highly developed assay technique, and the wider type of experimentation that will usually be preferable in the investigation of the properties of new and little known substances.

As a result of the preliminary work of Brande and Mitchell (1950) and Whittleston (1952), it was decided that the period for which milk might be expressed from the gland constituted the soundest measure of milk ejection activity. Moreover measures of flow rate and the volume secreted were

impractical with the equipment and labour available. Consequently after injection each sow had one or more teats milked and the time for which milk might be expressed from the gland was recorded. All milking was done by hand.

The exact standardization of the period of time between the prior suckling and the administration of pitocin proved to be an impractical procedure with the facilities available; however in the light of later work this objection to the technique was found to be of minor consequence only.

The period of time elapsing between consecutive injections in the one sow was fixed at 5 minutes. In the multi-injection schedules necessitated by the 4 point assay procedures, this period was reduced to 3 minutes; this practice being adopted in order that the total time for which the sow was in the bale was reduced. No discernable difference between serial responses to the two time intervals could be detected. The period of time between consecutive injection schedules on the one sow varied in accordance with the experiment being performed. Every alternate day was the maximum rate at which treatments were carried out.

THE PHENOMENON OF TACHYPHYLAXIS

The Derivation of the General Curve of Response with Successive Injections.

Early this century the pharmacological effects of posterior pituitary extracts were subject to a considerable amount of study. The pressor effect in particular engaged the attention of physiologists, amongst whom was Howell. Howell observed that when a number of consecutive doses of this extract, each being of the same size, were administered to an animal intravenously and in succession, there was, in effect, a rapid production of immunity, for the second injections and those thereafter produced a negligible response in terms of blood pressure rise. To this phenomena Schafer gave the name "Tachyphylaxis".

A similar effect has been observed in the case of lactating sows subjected to a continuous series of standard doses of the oxytocic substance. The time for which milk may be expressed from the gland, as a result of intravenous injection of the milk ejecting hormone, showed a steadily decreasing response with consecutive injections. This effect was common to all sows, irrespective of breed and was a remarkably constant feature of all of the observations made in this study.

Data illustrating the phenomena is presented in Table IV. (Fig.4.1, 4.2)

TABLE - IV

1. For all Sows ($\frac{1}{2}$ unit doses)

Number of Injections	1	2	3	4	5	6	7	8	9
Total of all responses (sec.)	6598	3228	3354	1021	1177	349	280	191	68
Number ofresponses	130	89	110	40	48	18	15	11	4
Mean (sec.)	50.8	36.2	30.5	25.6	24.5	19.4	18.6	17.4	17.0
Standard deviation	26.4	17.2	16.1	12.4	10.9	10.4	8.7	8.9	11.1
Coefficient of variation	51.9	47.5	52.8	48.4	44.4	53.6	46.8	51.1	65.3
Number of sows involved	Fourteen								

2. For Berkshire Sows only

Number of injections	1	2	3	4	5	6	7	8	9
Total of all responses	2419	1358	1675	390	696	222	192	167	45
Number of responses	61	42	55	15	28	9	9	9	2
Mean	39.6	32.3	30.45	26.0	24.8	24.7	21.1	18.5	22
Standard Deviation	11.8	10.3	10.0	3.8	3.2	3.7	9.6	-	-
Coefficient of variation	29.8	31.8	32.8	14.6	12.9	14.9	45.5	-	-
Number of sows involved	Six								

TABLE IV (Contd.)

3. For Tamworth Sows only

Number of injections	1	2	3	4	5	6	7	8	9
Total of all responses	3614	1515	1367	481	289	89	64	11.5	10.5
Number of responses	54	33	40	16	11	6	4	1	1
Mean	66.9	45.9	34.2	30.6	26.3	14.9	16.	11.5	10.5
Standard deviation	31.1	21.1	22.7	13.6	14.9	7.1	10.7	-	-
Coefficient of variation	46.5	45.9	66.3	44.4	56.8	47.6	66.8	-	-
Number of sows involved	Five								

4. For Large White Sows only

Number of injections	1	2	3	4	5	6	7	8	9
Total of all responses	565	355	312	150	192	38	24	13	11
Number of responses	15	14	15	9	9	3	2	1	1
Mean	37.6	25.3	20.8	16.6	21.3	12.6	12	13	11
Standard deviation	20.9	11.6	8.3	3.8	3.2	3.7	3.0	-	-
Coefficient of variation	55.6	45.8	39.9	22.8	15.0	29.3	25	-	-
Number of sows involved	Two								

An attempt has been made to fit an equation to this curve, for the expression of a suitable equation in general terms may provide an indication of the type of physiological process that lies behind the observed phenomena.

It was found that the equation

$y = a + be^{-cx}$ i.e. $dy/dx = c(a - y)$

provided an excellent fit. Where y = ejection time

x = number of ejections

a, b and c are constants.

This equation suggested that the rate of decrease of ejection time was proportional to the difference between the ejection time and a base time at any instant.

It is important to note that the results of any biological curve may be fitted by an infinity of mathematical functions, many of which though demonstrably inappropriate at the extreme ranges of response, are a good fit over the range used in practice. Faced with such a choice of functions, it is logical to adopt those which are of mathematical suitability, - which do not grossly violate any theoretical considerations as to the probable nature of the relationship existing.

In these respects the equation $dy/dx = c(a - y)$ is eminently suitable.

Demings method was used to find the numerical relationships involved in the equation. This involves an approximation series correcting consecutively estimated values of a b and c. A fairly accurate first point approximation was obtained by picking three points lying as close to the expected curve as possible and having equal increments of x.

If these three points $(x_1\ y_1)$ $(x_2\ y_2)$ $(x_3\ y_3)$ are selected such that $x_1 + x_3 = 2\ x_2$ then

$$a_o = \frac{y_1\ y_3 - y_2^2}{(y_1 + y_3) - 2y_2}$$

$$c_o = 2.30258 \log_{10} \frac{y_1 - a_o}{y_2 - a_o}$$

$$b_o = \text{antilog} \quad \log_{10} (y_1 - a_o) + 0.43429\ c_o x$$

These values of a_o, b_o, c_o were progressively corrected in the manner described by Deming, until the correction terms approached zero.

Using this technique the equation $dy/dx = c\ (a - y)$ took the following value

$$y = 15.313 + 49.102\ e^{-0.379x}$$

and using this as a prediction equation we have the following result:

Number of injections			3	4	5	6	7	8	9
Actual mean response	50.8	36.2	30.5	25.6	24.5	19.4	18.6	17.4	17.0
Predicted response.	48.9	38.3	31.1	26.1	22.7	20.4	18.8	17.7	16.9

(Fig.4.1)

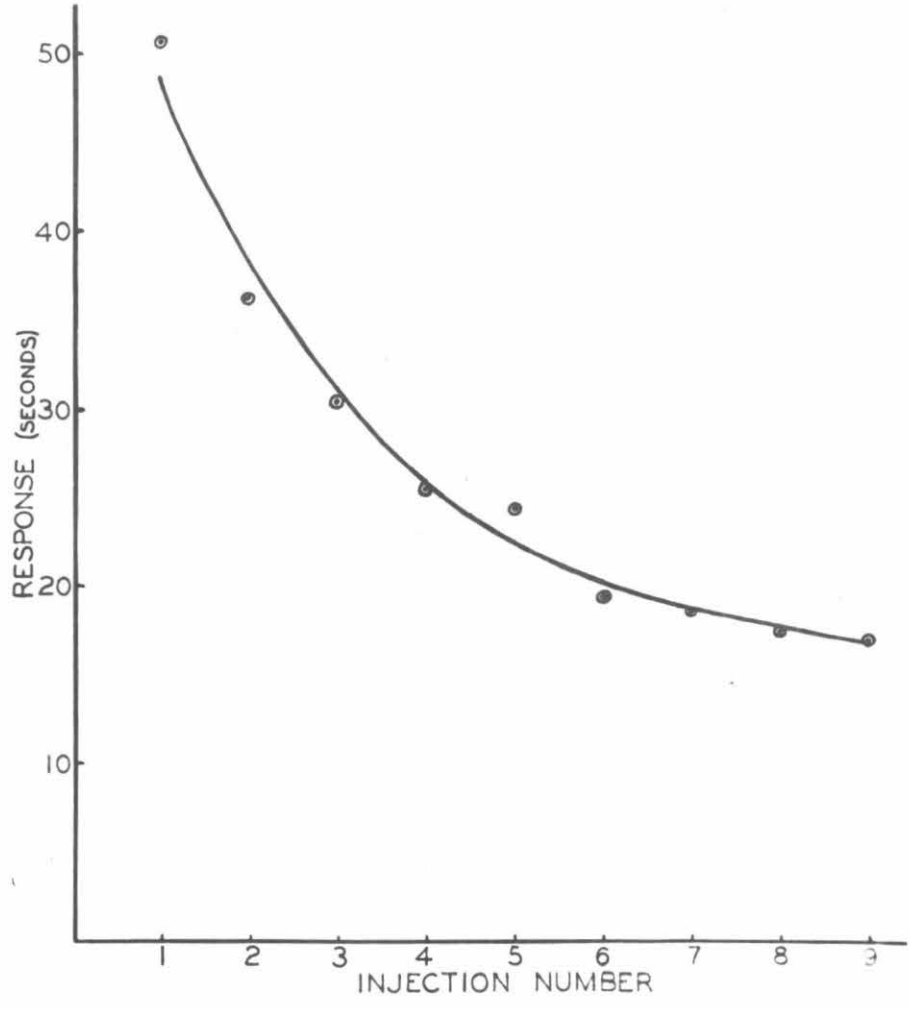


Fig.4.1 - The curve $y = 15.313 + 49.102e^{-.379x}$, together with the average milk ejection response recorded for all breeds to consecutive standard doses of pitocin.

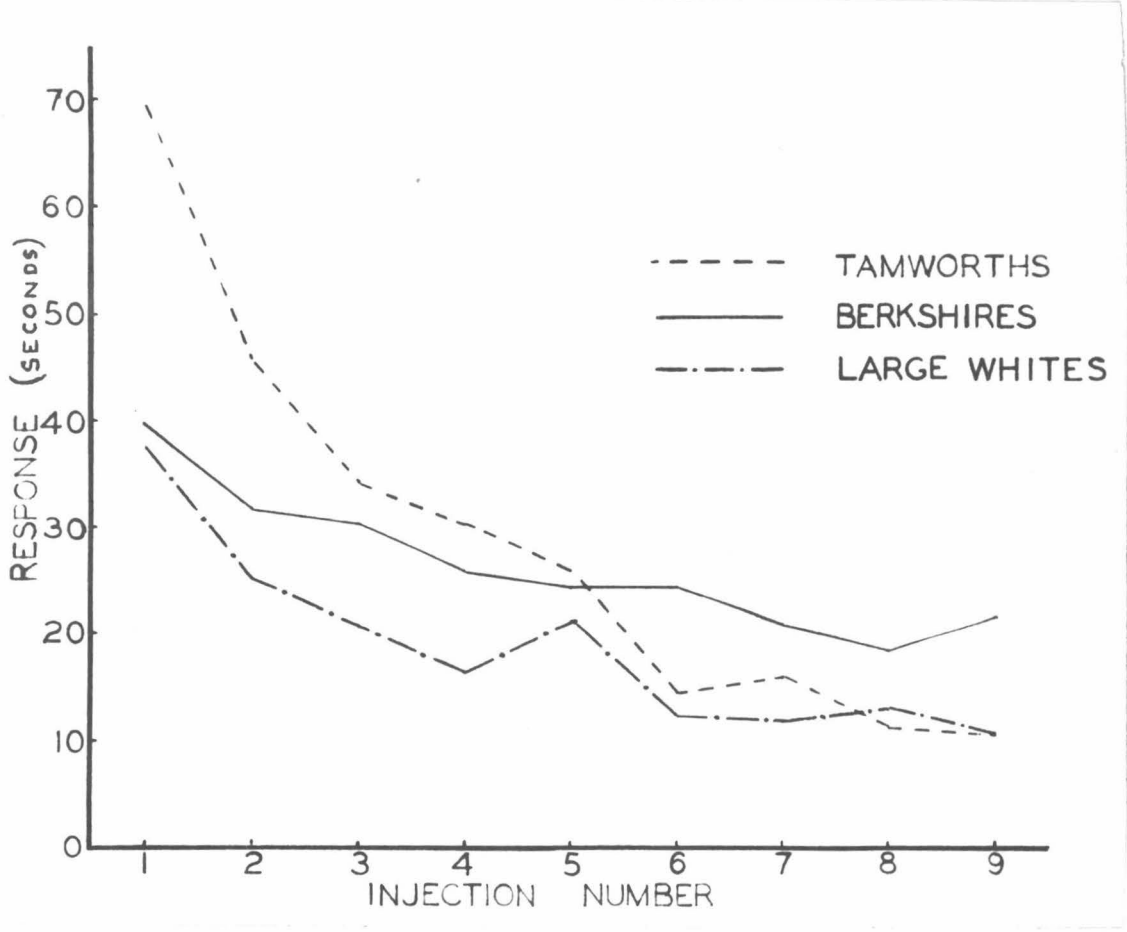


Fig.4.2 - The average milk ejection response for individual breeds recorded over nine consecutive standard doses of pitocin.

This equation is of considerable value in that it gives us a value of a_0 i.e. the point of inflection of the curve or base time, whereby knowing the response at two times t_1 and t_3, an estimation of response to a standard dose at the middle time t_2 may be derived.

$$y_2 - a = \sqrt{(y_1 - a)\ (y_3 - a)}$$

This estimation is necessary if a one point assay is to be successful. (See One Point Assays)

In the data presented in Table IV it may be seen that the responses of the Tamworths and Berkshires are markedly different. The difference between the Large Whites and Berkshires is very much smaller; however, to establish that this difference is significant means little for only two large white sows were available. Any generalisation from a population so limited in representation is, to all intents and purposes, quite worthless. For this reason the regression equations of the three breeds have not been compared for statistical significance.

The declining number of responses recorded for successive injections is merely a function of both the difficulties of multi-injection schedules encountered at the commencement of this study, and the source of the data presented. A series of half unit injections constituted a portion of many experiments performed. Where such injections,

uncomplicated by further treatments, have been carried out, the resulting responses have been summarised and used in this table. Standard deviations and coefficients of variation of recorded responses where small in number have not been determined, as a figure so determined, is of little value. Thus the blanks in the table.

A Discussion of the Phenomenon and its Implications

The apparent breed difference in sensitivity is an interesting point for speculation. In a series of pilot observations made by the author at the Massey piggery, no marked difference in suckling time between breeds could be detected. If this observation is correct, the variation in sensitivity indicates that variations in the output of the milk ejection factor, in response to the stimulation of suckling, exist between breeds. It has been noticed in these observations that the sow ceases to let-down milk in spite of continued suckling and worrying of her piglets. If this cessation of let-down is due to the complete exhaustion of the milk ejection factor, it follows that the yield of milk will be limited by the production and availability of this factor. In turn it follows that the greater the sensitivity of the gland, the less important the availability of the let-down factor is likely to be, with a consequent decline in the chance that milk yield will be affected.

It is conceivable that a similar situation

might exist between breeds of dairy cows.

The cause of the variation in sensitivity is obscure. In view of the fact that pressure influences the period of let-down (Page 98) it may be logically suggested that a breed difference exists in the average milk content of the mammary glands, thereby influencing the normal level of mammary pressure, which in turn influences the response to a standard dose of the let-down hormone. On the other hand the explanation may be considerably more complicated than this simple hypothesis would suggest, involving such complex problems as cell membrane permiability and enzyme activity.

In a private communication, Turner (1952a) sought to explain the phenomena of tachyphylaxis in terms of pitocinase activity, postulating that the administration of pitocin causes an increase in the activity of pitocinase with a consequent decrease in reaction time. That such a hypothesis is probably incorrect is demonstrated on Page 127. Whittleston (1952) explained the phenomena in terms of an increasing "threshold" doseage being required by the myoepithelium before contraction ensued.

An alternative explanation appears to lie in terms of mammary pressure effects - the removal of milk with each subsequent dose lowering the pressure,

and in consequence reducing the period of response. The flattening out of the curve of response with time might well be explained in terms of the inactivating mechanism being gradually impeded in effeciency - as the data presented in the final section of this thesis would suggest, so providing a compensating mechanism for the general decrease of response with declining pressure. Thus, though the efficiency of the action of the hormone decreases, its rate of inactivation declines, resulting in a response that is reasonably constant.

The phenomenon of tachyphylaxis assumes significance when considering the efficiency of milking. The double let-down of milk in lactating dairy cows at any one milking is a comparatively common occurrence (Whittleston 1952a). In such a case, the release of a second amount of the let-down hormone would appear to result in a diminished let-down response in that animal. If then the storage and production of the let-down factor within the body is strictly limited, then because of the diminished availability of the let-down factor, the following milking is likely to be impaired, thus making the stimulation of double let-down a necessity if the maximum yield is to be obtained. While such a hypothesis is strictly speculative, it is conceivable that an equilibrium might arise between the supply of

the let-down factor and the necessity of double let-down. This equilibrium may be such as to limit the total yield at any one milking, resulting in an overall decrease in milk production.

THE BIOLOGICAL ASSAY OF THE MILK EJECTION HORMONE

Prologue

The biological assay of physiologically active substances is a necessary procedure when the substance concerned has not been isolated as a crystalline compound, or when the amounts involved are so small that they defy chemical definition. Attempts have been made to measure such a substance in terms of its physiological effect, with the consequent definition of a unit in terms of a quantitative biological response - such a definition implying that biological properties are not variable. Mouse units for oestrin, frog units for digitalis, the rabbit unit for progesterone and dog units for cortical extracts are typical examples of these early attempts at measurement - all of which were quantitatively valueless. Each unit, defined in this manner is very variable - this variation being due to technique, to environment, and to inherent individuality.

In contrast to these results, it has been found that a comparison of the potency of two preparations having the same specific action gives constant results regarding the ratio of the respective potencies. The introduction of standards in dry stable form makes comparative assays feasible, and an estimation of potency with reference to the standard

makes possible a procedure leading to an accurate and repeatable result.

The standard of pituitary (posterior lobe) extract is a quantity of powder prepared from fresh posterior lobe material by treatment with acetone and subsequent drying over phosphorus pentoxide. This preparation is held at the National Institute for Medical Research, London, being stored and distributed in ampules kept at 0°C. By agreement, one international unit is the amount of activity contained in 0.5 mg of powder when this powder is extracted in the prescribed manner.

Three of the four major pharmacological properties of the posterior pituitary now have recognised assay procedures. Pressor activity is assayed in terms of a rise of B.P. (Blood Pressure) in an anaesthetized animal after intravenous injection of the extract. The antidiuretic effect is measured in terms of the antidiuresis and urinary chloride concentration shifts, in intact unanaesthetized animals, while the oxytocic extract is assayed in terms of the contraction of uterine muscle "in vitro", or alternatively, by the depression of blood pressure in an anaesthetized bird. The fourth major physiological effect, namely milk ejection, is as yet without an accurate and simple assay procedure. It is desirable to rectify this situation, for without a standard assay technique, studies of the

physiological role of this hormone are seriously impaired.

The purpose of this work was to develop a relatively simple, accurate and reliable procedure for the biological assay of the milk ejecting activity of extracts of the posterior lobe, or other sources of this substance, such a method to permit an objective estimate of the reliability of each determination.

The Derivation of the Method

Whittleston (1952) suggested a method based on intravenous injection of the posterior pituitary extract into a lactating sow, eliciting an almost immediate, marked and transitory ejection of milk. His procedure consisted of injecting standard and unknown solutions alternately until doses of the two producing equivalent periods of milk ejection were determined. Although the test possesses the advantages of simplicity and economy, two objections may be noted. First, the method leaves to the discretion of the assayist the decision as to which responses may be considered equal, for periods of ejection which are equal are unusual, while the interpretation is further complicated by the phenomena of tachyphylaxis . The second objection is the difficulty of computing the error of each assay, for such an estimate is impossible using this technique.

The preliminary data of Brande and Mitchell (1949) indicated that a linear relationship existed between log dose of pitocin administered and the response in terms of the period of milk let-down. (Fig. 5.1)

Dose in I.U.	1	3	5	10
Time of flow (secs.)	41	78	82	104

If the relationship suggested by these workers' results could be substantiated and applied over a range of doses considerably smaller than those they used, it is a comparatively simple step to outline in theory an assay technique - a technique having three maxims; it must be simple, it must compare the standard and unknown, and it must be reproducible (Burn 1950).

It we choose two doses of the standard and two doses of the unknown in the same ratio, the standard and unknown being of approximately the same potency as determined by preliminary assay such that

s_1 is the smaller dose of the standard

s_2 is the larger dose of the standard

u_1 is the smaller dose of the unknown

u_2 is the larger dose of the unknown

$$\text{then } \frac{s_2}{s_1} = \frac{u_2}{u_1}$$

and if the means responses are f_1 f_2 f_3 and f_4 respectively the mean response to the two high doses

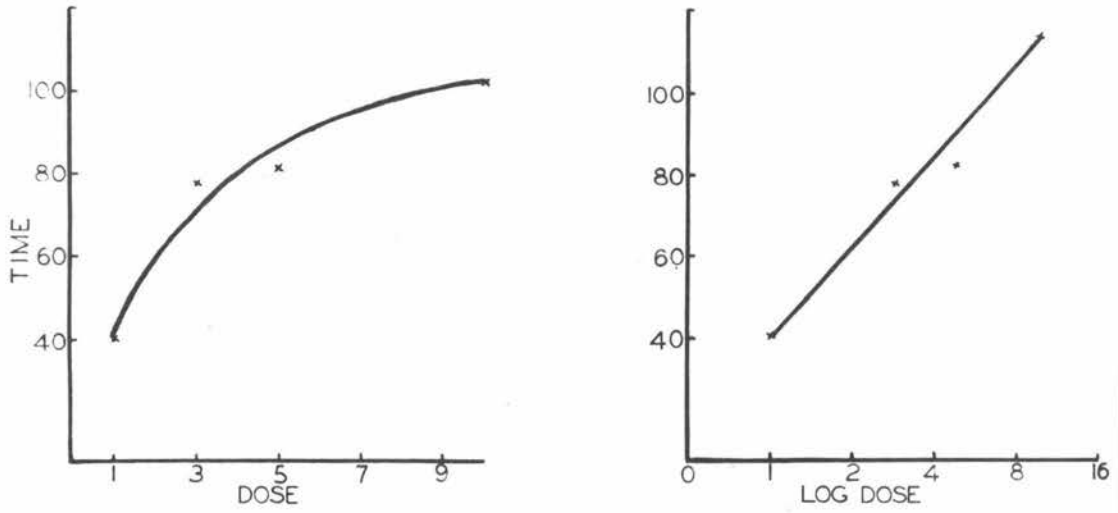


Fig. 5.1 - The dose response, and log dose response curves as plotted from the data published by Brande and Mitchell.

is $\frac{f_2 + f_4}{2}$ and the mean response to the two low doses is $\frac{f_1 + f_3}{2}$. The difference between these i.e. $\frac{f_2 + f_4}{2} - \frac{f_1 + f_3}{2}$, is the mean effect of increasing the dose by s_2/s_1 - represented by c.

In effect this gives a measure of the log dose response line for it measures the effect of increasing the dose on response.

A measure of the difference in response to the high doses minus the difference in response to the low doses gives a measure of the difference in the response of the standard + the unknown i.e. $\frac{(f_2 - f_4) + (f_1 - f_3)}{2}$ represented by d. Then as the response is proportional to log dose $\frac{c}{d} = \frac{\log s_2/s_1}{m}$ where m = log ratio of the potency of the two preparations.

m may thus be determined.

Experiments were designed to test this hypothesis. The derivation of the dose response curve was the first problem followed by the application of the principle as outlined above and made possible by this linear relationship.

Experimental - The Dose Response Curve

A latin square design using four sows and four treatments formed the basis of these investigations. It was also desired to investigate the possible use of one point assays. Earlier experiences indicated that the second, third or fourth injection in a half unit series might prove the most suitable for such an assay; thus it was decided to administer the varying dose at the second point in one series, and at the fourth point in a further series. The experiment was as follows :

Trial I

Square	Sows	Treatments
A B C D	Pam	½ - 1/4 - ½ units C
B A D C	Gladies	½ - ½ - ½ " D
C D B A	Fala	½ - 1 - ½ " A
D C A B	Peg	½ - 2 - ½ " B

Doses were administered at approximately 3 minute intervals, the response measured being the period of actual milk let-down. Three sows, Pam, Fala and Peg were Tamworths, Gladies a large white.

Trial II

Square	Sows	Treatments
A B C D	Pam	½ - ½ - ½ - 1/4 - ½ units A
B A D C	Gloria	½ - ½ - ½ - ½ - ½ " B
C D B A	Fiona	½ - ½ - ½ - 1 - ½ " C
D C A B	Fancy	½ - ½ - ½ - 2 - ½ " D

Pam and Fancy were Tamworths, Fiona a Berkshire and Gloria a large white. Again doses were administered

at approximately 3 minute intervals, and the response measured in seconds.

Results

Trial I

75	72	14	53	62	3	31	11	27	20	16	14
40	78	21	37	36	16	65	37	26	23	10	10
43	16	22	51	32	27	45	75	11	18	37	15
82	65	39	68	19	53	72	56	28	40	71	24

Trial II

60	60	50	28	45	90	60	50	180	40	87	61	56	50	45	90	58	39	82	32
60	25	25	90	23	58	34	34	25	28	60	35	28	60	25	40	31	28	27	28
65	55	50	55	60	60	49	50	95	30	39	30	25	48	18	29	25	27	12	20
55	35	20	60	20	84	40	42	39	36	85	75	35	17	33	50	45	40	85	15

Sorted into treatment effects these give -

Trial I	A	B	C	D	Trial II	A	B	C	D
	$\frac{201}{4}$	$\frac{286}{4}$	$\frac{56}{4}$	$\frac{150}{4}$		$\frac{82}{4}$	$\frac{403}{4}$	$\frac{171}{4}$	$\frac{297}{4}$

giving the mean responses

	1/4 unit	½ unit	1 unit	2 units
Trial I	14.0	37.50	50.25	71.50
Trial II	20.5	42.75	74.25	100.75

These results indicate a straight line relationship between log dose and response. (Fig. 5.2).

As a check on these results, both trials were repeated using the same 4 Berkshire sows in each, the object being to reduce the initial variation due to breed differences.

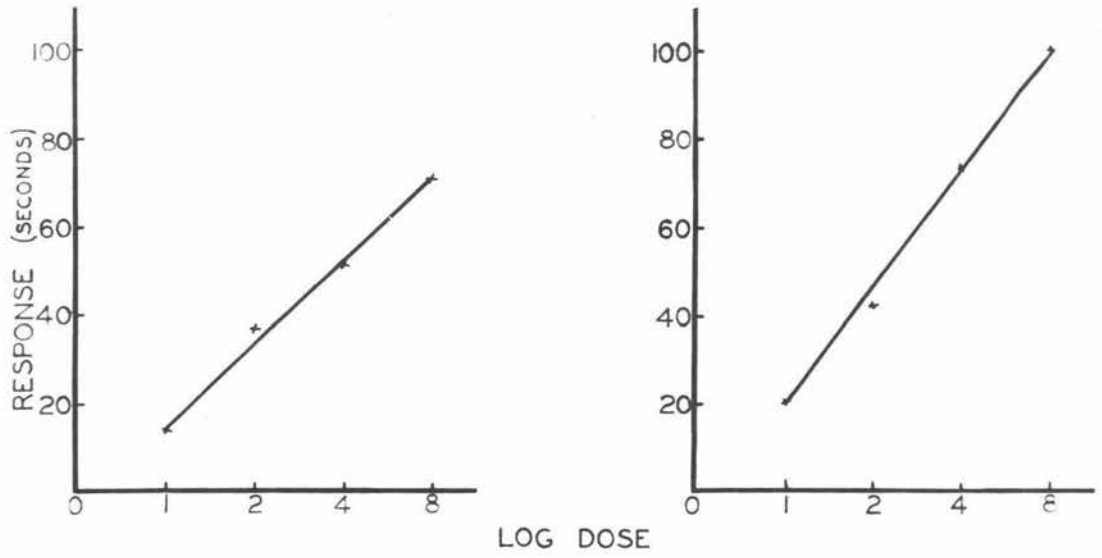


Fig. 5.2 - The log dose response line as plotted from the results of Trial I and Trial II.

Trial III

A	B	C	D	Fay	½ - 1/4 - ½ C
B	A	D	C	Fatima	½ - ½ - ½ D
C	D	B	A	Ella	½ - 1 - ½ A
D	C	A	B	Felecia	½ -2 - ½ B

Trial IV

A	B	C	D	Fay	½ - ½ - ½ - 1/4 - ½ A
B	A	D	C	Fatima	½ - ½ - ½ - ½ - ½ B
C	D	B	A	Ella	½ - ½ - ½ - 1 - ½ C
D	C	A	B	Felecia	½ - ½ - ½ - 2 - ½ D

Results

Trial III

45	74	36	33	95	27	30	14	25	34	30	28
60	137	55	43	86	34	43	39	41	40	12	15
53	20	47	35	32	32	51	43	32	38	90	41
51	42	37	49	31	43	57	60	20	39	95	21

Trial IV

30	30	30	16	28	40	95	30	28	32	32	30	30	42	18	30	20	18	70	16
44	42	36	34	23	35	29	30	18	23	24	17	16	37	14	25	20	18	32	14
40	30	30	55	30	40	36	30	80	20	32	30	28	27	24	30	29	30	15	27
37	32	32	90	18	54	47	45	78	35	43	36	30	14	27	50	54	41	41	50

Sorted into treatment effects these give -

Trial III

C	D	A	B
77/4	143/4	310/4	440/4

Trial IV

A	B	C	D
63/4	130/4	207/4	277/4

giving the mean responses

	1/4 unit	½ unit	1 unit	2 units
Trial III	19.75	35.75	77.75	110
Trial IV	15.75	32.50	51.50	69.25

Again the 4 points are seen to lie about a straight line. Hence it was concluded that further investigation of the proposed assay technique was warranted. (Fig.5.3)

The Four Point Assay

The attempted assay procedure was as follows :

A lactating sow was prepared in the usual manner. 0.5 cc of pitocin (1 cc = 10 units) was diluted with 4.5 cc of physiological saline. The extract to be tested was diluted with the same dilutent in such a manner that it was expected to be equal in potency to the diluted standard solution (s_1). Twice the amount of s_1 was designated as the high dose of the standard (s_2). An amount of diluted unknown solution approximately equal in concentration to the low dose of the standard was taken as the low dose of the unknown (u_1) and an amount exactly twice this in volume or concentration was the high dose of the unknown (u_2). The injection schedule was set up by assigning the various doses at random to a 4 x 4 Latin square so that the four doses were each administered four times, making a total of sixteen injections.

Certain difficulties are involved in carrying out this procedure. These may be summarized as follows :

1. A quiet sow is required, together with an operator of sufficient skill to administer sixteen consecutive intravenous injections.

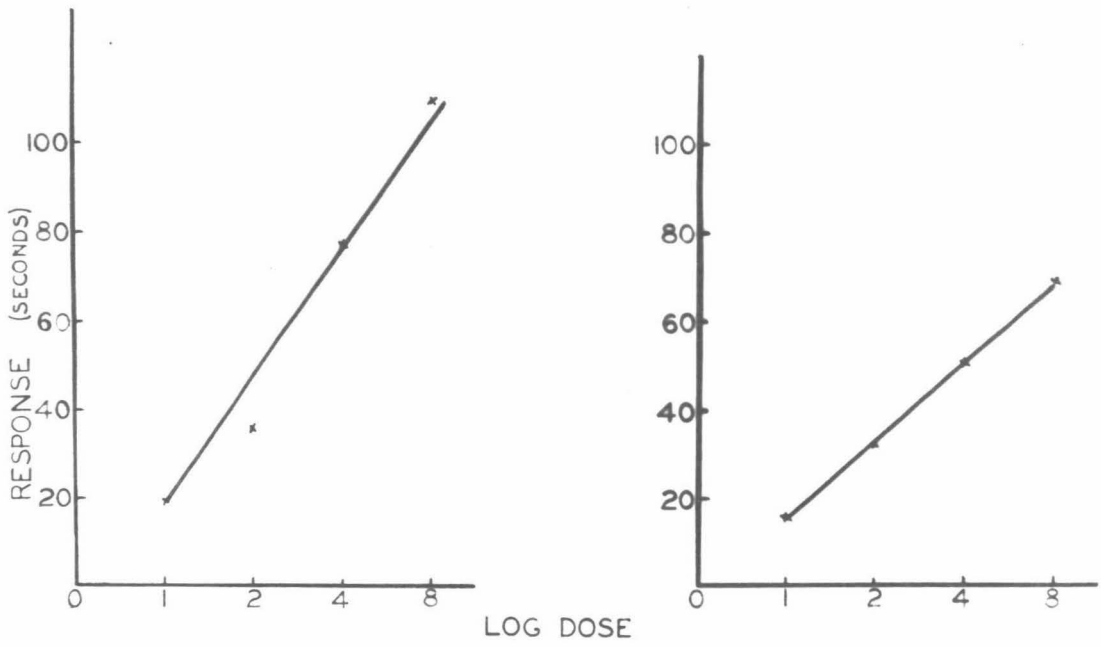


Fig. 5.3 - The log dose response line as plotted from the results of Trial II and Trial IV.

2. A preliminary estimate of the strength of the unknown solution needs to be made by preliminary matching of the known and unknown on a different sow. It is obvious that the larger the difference between them, the larger is the length of the regression line which is used in the calculation of potency, with a consequent less precise result.

3. The doses need to be made up with great precision.

4. The rapid development of tachyphylaxis may invalidate the assay when milking is carried out on only one teat. This problem may, if necessary, be overcome by changing either the teat milked, or the size of the dose. In doing so, the relationship among the various doses as set forth must be maintained, while doses and/or teats may be changed only at the beginning of a new row of the injection schedule.

Protocol of Assay

Sow - Peg

Date - 26th January

Square					
	A	B	C	D	A = s_1
	B	A	D	C	B = u_1
	C	D	B	A	C = s_2
	D	C	A	B	D = u_2

Result

s_1	u_1	s_2	u_2
60	47	92	90
u_1	s_1	u_2	s_2
42	40	85	80
s_2	u_2	u_1	s_1
78	65	31	25
u_2	s_2	s_1	u_1
60	55	28	24

Totals

s_1	s_2	u_1	u_2
144	305	153	300

The mean of the two mean results obtained with the high doses C + D is the mean of $\frac{300}{4} + \frac{305}{4}$ i.e. 75.625.

The mean of the two mean results obtained with the low doses A + B is the mean of $\frac{144}{4} + \frac{153}{4}$ which is 24.625.

Thus 75.625 - 24.625 i.e. 51 is the mean increase in response produced by doubling the dose.

The difference in the effect of A + B is 2.25

The difference in the effect of C + D is 1.25

these differences being in opposite directions, hence the mean difference between standard and unknown is 0.25.

As the period of ejection is proportional to log dose it follows that

$$\frac{51}{0.25} = \frac{\log 2}{M}$$

Where M = log of ratio of the potency of the two preparations in the doses injected.

hence $M = \frac{0.301 \times 0.25}{51} = \frac{0.07525}{51} = 0.014754$

the antilog which is 1.035.

Thus since the unknown produced a greater mean ejection response than the standard

$$\frac{B}{A} = 1.035.$$

Hence 1 cc of the unknown contains 10.35 units. As the unknown and standard were identical the estimate is in error by $\frac{10.35 - 10}{10} \times \frac{100}{1}\%$ i.e. 3.5%.

While this result may be considered satisfactory, a more detailed analysis of the responses is capable of producing a more accurate result with the added advantage of allowing an estimate of the standard error of the assay result. This analysis was outlined by Emmens (1950).

The analysis is again dependant upon a linear relationship between log dose and response. If two dose response lines are plotted, one for the known the other for the unknown, and if the two are parallel, the two lines may be regarded as two separate estimates of a common slope relating response to the dose of both preparations. The pooling of this information from both samples makes it possible to calculate one value of b in the equation

$E = a + bx$ where E is the estimate of response. The relative potency of the two preparations then depends on the difference between the two mean doses,

and the difference between the two mean responses to all doses. Thus if M is the log ratio of the potency of the unknown to that of the standard then

$$M = \overline{x_s} - \overline{x_u} + \frac{\overline{y_u} - \overline{y_s}}{b}$$

where $\overline{x}_u$ is the mean log dose of the unknown and $\overline{x}_s$ is the mean log dose of the standard, $\overline{y}_u$ the mean response to the unknown and $\overline{y}_s$ the mean response to the standard.

In balanced designs of this type where equal numbers of doses of the standard and unknown, at geometric doseage levels, are given to groups of equal numbers of animals, the analyses of the dose response lines may be simplified by the use of factorial coefficients. Using 2 doses of the known and 2 of the unknown there are three degrees of freedom and thus three independant comparisons which may be made by factorial analysis. The three sources of variation concerned are those due to differences between the response to the two samples, to linear regression, and to departure of the two dose response lines from parallelism.

Protocol of Assay

Sow Peg, 26th January.

Square							
	A	B	C	D	A	S_1	$S_1 = U_1 = \frac{1}{2}$ unit pitocin
	B	A	D	C	B	U_1	$U_2 = S_2 = 1$ " "
	C	D	B	A	C	S_2	
	D	C	A	B	D	U_2	

Table I

s_1	u_1	s_2	u_2	
60	47	92	90	289
u_1	s_1	u_2	s_2	
42	40	85	80	247
s_2	u_2	u_1	s_1	
78	65	31	25	199
u_2	s_2	s_1	u_1	
60	55	28	24	167
240	207	136	219	902

s_1	s_2	u_1	u_2
144	305	153	300

Table II

Factorial Coefficients

	u_1	u_2	s_1	s_2	d	S.P.	Variance	S.D.
Samples	-1	-1	+1	+1	16	-4	1	
Slope	-1	+1	-1	+1	16	308	5929.00	
Parallelism	-1	+1	+1	-1	16	-14	12.25	
Totals	153	300	144	305			5943.25	

The relevant factorial coefficients are inserted in Table II, the sum of products computed using the coefficients and the totals listed from Table I. From the sums of products, the variance

attributable to each source of variation is the square of this amount divided by the divisor (a), and with it is associated a single degree of freedom. The sum of squares for random sampling is the error sum of squares; the mean square for error, in this case 19.25 is written at the foot of the variance column. A normal analysis of variance follows and the importance of each source of variation examined by the usual F test.

Source	SS	df	MS	F	Results
Rows	2154.75	3			
Columns	176.25	3			
Samples	1.00	1	1 D^2	1	N.S.
Slope	5929.00	1	5929 B^2	308	**
Parallelism	12.25	1	12.25	1	N.S.
Error	118.50	6	19.25 S^2		
Totals	8391.75				

The mean square for the difference between samples, slope and parallelism is the same as the Treatment mean square. The mean square of the samples is denoted by D^2, that of slope by B^2. The F value of D^2 shows whether the potency of the actual doses of the unknown which were administered differed from the standard. The variance attributable to linear regression i.e. B^2, measures the average increase in response due to equivalent increases in the doses of the standard and the

unknown as determined by the combined slope. B^2 must be significantly greater than the error variance or the assay is not valid, since the slope does not differ significantly from zero. Our present B^2 gives an F of 308 and shows that the value of the slope is in fact highly significant, a point not apparent from preliminary analysis of variance in II. This high significance of B is found because the variances attributable to the various departures from linearity and parallelism are particularly small.

Computation of the log ratio of potency using factorial coefficients is enabled by the use of the formulae (Emmens 1950)

$$M = \bar{x}_s - \bar{x}_u + \frac{kID}{B}$$

where k = I for a 4 point assay.
I = interval in logs between successive doses
D and B the square roots of D^2 and B^2. These are given the sign of the sums of products from which they were originally computed. The terms k + I convert the log potency from the answer given by factorial coefficients back to normal logarithms.

In order to find the potency of the unknown in terms of the number of units it contains per cc, we may assume that both the standard and unknown are of equal potency and that both are administered in the same doseage units and thus

$$\bar{x}_s = \bar{x}_u$$

and the equation reduces to $M = \frac{k\ I\ D}{B}$

The antilog of M is the number of units of the standard required to give the same response as one assumed unit of the unknown. The highest dose of the standard was one unit, our assumed unit for the unknown is thus unity and equivalent to 1 cc pitocin.

Substituting in the equation we find that

$$M = \log 10 \text{ potency u/s} = \frac{1\ D}{B}$$

$$= \frac{0.30103 \times 1}{5929}$$

$$= 0.00391$$

Thus the log of the potency of 1 ml of the unknown is .00391, the antilog of which is 1.009. Hence 1 cc contains 1.009 units.

The standard error of M Sm is approximately given by the formulae

$$Sm = S\ k\ I \quad B^2 + c$$

where S = root

Sm is used in conjunction with a table of "t", with n, the number of degrees of freedom equal to the number of degrees of freedom for experimental error in the analysis of variance. The value of t for any required degree of significance, usually P = 0.05, is read from the table and multiplied by Sm; then the potency of the unknown preparation has been determined within the limits of antilog (M + t Sm) and antilog (M - t Sm). These limits are approximate only for they are

derived from a formulae which closely approaches the exact formulae only if the slope of the dose response line has been determined with little error.

For this assay

$$Sm = \frac{S\,k\,I\,\sqrt{B^2 + D^2}}{B^2} = \frac{19.75\ (.30103)\ 5930.00}{5929}$$

$$= 0.1738$$

The 95% limits of M are $.00391 \pm (2.447)(.01738)$

i.e. $.00391 \pm .04253$

i.e. T	.96138	.00391	.04644
the antilog of which gives	.915	1.009	1.113

Discussion

In the method of calculation presented above certain potential sources of variation are ignored. These are the influence of the magnitude of response immediately preceding a given response, and the influence of the size of the preceding dose. The former may be minimized by giving one or two preliminary ½ unit injections and discarding the response as far as the assay is concerned while the design of the injection schedule is such as to minimise effects due to size of the preceding dose. Variation due to tachyphylaxis is similarly accounted for by the design and the method of analysis. Using two or four teats, i.e. one per row of the injection schedule, will almost certainly decrease the error term by reducing tachyphylaxis and thereby increasing the difference between doses.

A total of 6 four point assays have been carried out, the results of which are tabulated below. A 6 point assay has also been tried but appears to offer no advantage over the four point assay for in this case two animals must be used.

Assay of Solutions of Known Potency - A Summary of Results

Assay No.	True Potency	Potency Found	Standard Error Range		Actual Error as % of True Potency
1	1.0	.95	0.5	1.8	4.5%
2	1.0	.88	0.7	1.0	11.5%
3	1.0	1.01	0.9	1.1	1.0%
4	0.69	0.71	0.6	0.8	3.2%
5	0.83	0.85	0.8	0.9	2.2%
6	0.60	0.56	0.3	0.9	6.6%
7* (6 point assay)	0.66	0.70	0.5	1.0	6.1%

Burn in his standard work on Biological Assay states :

"Methods are good if they are accurate, rapid, and simple, and bad if they are inaccurate, slow and need skill. That accuracy is necessary all are agreed. That speed in obtaining a result is important is well known to those engaged in estimations on behalf of commercial firms. Still fewer people realise that methods are good in proportion as the technique is simple."

The method outlined for the assay of the milk ejection hormone fulfils these requirements. The

technique of the assay is simple, requiring only the ability to handle a sow and to give an intravenous injection. Although the mathematics underlying the method of computation are intricate, their understanding is not necessary to the assayist who has only to substitute in the appropriate formulae and use arithmetic to calculate the results.

SUMMARY

1. Data is presented illustrating that the regression of response on log dose is linear.

2. The demonstration of this fact allows a method of assay to be undertaken giving good results, and allowing an estimate of the standard error of each estimation to be made.

A ONE POINT ASSAY FOR THE MILK EJECTION HORMONE

The Problem

The development of an accurate assay technique for the milk ejection hormone is a matter of considerable importance in making a study of the physiology of milk secretion. Without an assay method quantitative studies in many fields are impaired or denied. The use of a four point assay offers the possibility of assessing the activity of any extract, and is therefore a technique of considerable value. However, it suffers from the disadvantage of requiring at least 3 units of the extract, and this quantity may be sufficiently large to render the assay unpracticable. The development of a one point assay using as little as 0.25 units would offer a real advantage in allowing the problems of milk ejection to be further elucidated. The vicissitudes of biological material makes the possibility of a quantitatively accurate one point assay a doubtful proposition, yet with the application of mathematical analysis an approach to the problem is made possible.

The period of response in the lactating sow to consecutive doses of Pitocin shows a steady decline in magnitude and in variability. Whilst sensitivity may persist, the differentiation of small doses with small responses becomes increasingly difficult, thus the problem is one of equating the magnitude of response with

the variability of response, for the point where maximum dose differentiation can be most readily detected is the ideal assay point. The expression, coefficient of variation, i.e. the percentage ratio of the mean response at any one point to the standard deviation of responses at that point, ideally indicates the preferred point, being subject to modification however by the practicability of a series of half unit doses in a capricous sow.

Experimental

An analysis of preliminary data indicated that the coefficient of variation for each consecutive standard injection was relatively stable. In the absence of alternative information, it was decided to use the second and fourth injection points as those subjected to dose variability, while following the variable dose with a further half unit dose in order that an estimate might be made of the likely importance of the size of the preceding dose on the succeeding one. If it could be shown that this effect was negligible, the opportunity existed of using the responses to standard doses, administered before and after the unknown, in order to estimate what the response would have been at the point where the unknown was given, had a standard dose been given in its place, for it has already been shown that knowing the responses y_1 and y_3, y_2 can be calculated from the formulae

$$y_2 - 15.0 \doteq \sqrt{(y_1 - 15)\,(y_3 - 15)}.$$

Obviously a comparison of response of the unknown and the standard, at the same injection point, is the logical basis for any one point assay.

A Latin square design using four sows, four treatments and four repetitions, formed the basis of the trials. The four sows were subject to treatment on alternate days, one row of the square being carried through each day of treatment, thus each trial lasted eight days and involved 48 injections.

Trial I

2nd point variable.

Square	Sows	Treatments	
A B C D	Pam	½ - 1/4 - ½ units	C
B A D C	Gladies	½ - ½ - ½ "	D
C D B A	Fala	½ - 1 - ½ "	A
D C A B	Peg	½ - 2 - ½ "	B

Result

Fala			Pam			Peg			Gladies		
75	72	14	53	62	8	31	11	27	20	16	14
40	78	21	37	36	16	65	37	26	23	10	10
43	16	22	51	32	27	45	75	11	18	37	15
82	65	39	68	19	58	72	56	28	40	71	24

Trial II

4th point variable.

Square	Sows	Treatments	
A B C D	Pam	½3 - 1/4 - ½ units	A
B A D C	Gloria	½3 - ½ - ½ "	C
C D B A	Fiona	½3 - 1 - ½ "	D
D C A B	Fancy	½3 - 2 - ½ "	B

Result

Pam					Gloria					Fancy					Fiona				
60	60	50	28	45	90	60	50	180	40	87	61	56	50	45	90	58	39	82	32
60	25	25	90	23	58	34	34	25	28	60	35	28	60	25	40	31	28	27	28
65	55	50	55	60	60	49	50	95	30	39	30	25	48	18	29	25	27	12	20
55	35	20	60	20	84	40	42	39	36	85	75	35	17	33	30	45	40	87	15

Analyses of Variance were carried out on the three responses of Trial I, and the five of Trial II.

Trial I -

1. First Injection (all treatments the same)

Source	S.S.	df	M.S.	F	Result
R	1754.19	3			
C	2827.19	3			
Tr	423.69	3	141.23	1	N.S.
E	1058.37	6	176.39		
T.	6063.44	15			

2. Second Injection (treatments 1/4, $\frac{1}{2}$, 1 and 2 units)

Source	S.S.	df	M.S.	F	Result
R	475.19	3			
C	1373.19	3			
Tr	6942.69	3	2314.23	28.66	* *
E	484.37	6	30.73		
T.	9275.44	15			

3. Third Injection (Treatments - residual effects only)

Source	S.S.	df.	M.S.	F.	Result
R	1181.00	3			
C	282.50	3			
Tr.	487.54	3	162.50	2.37	N.S.
E	411.13	6	68.52		
Ts.	2362.07	15			

Trial II

1. First Injection (all treatments the same)

Source	S.S.	df.	M.S.	F.	Result
R	2685.5	3			
C	987.4	3			
Tr.	327.5	3	109.1	1	N.S.
E	1516.6	6	251.1		
Ts.	5517.0	15	367.8		

2. Second Injection (all treatments the same)

Source	S.S.	df.	M.S.	F.	Result
R.	819.1	3			
C.	218.2	3			
Tr.	165.0	3	55	1	N.S.
E.	2276.7	6	379.4		
Ts.	3479.0	15			

3. Third Injection (all treatments the same)

Source	S.S.	df.	M.S.	F	Result
R.	855.7	3			
C	248.	3			
Tr.	245	3	8.16	1	N.S.
E.	854	6	14.2		
Ts.	2204	15			

4. Fourth Injection (Treatments 1/4, $\frac{1}{2}$, 1 and 2 units)

Source	S.S.	df.	M.S.	F	Result
R.	346.32	3			
C.	380.47	3			
Tr.	1488.27	3	496.09	8.08	*
E.	368.14	6	61.36		
T.	2583.30	15			

5. Fifth Injection (Treatments - residual effects only)

Source	S.S.	df.	M.S.	F	Result
R.	564.7	3			
C.	381.3	3			
Tr.	775.3	3	287.65	4.22	N.S.
E.	408.5	6	68.08		
T.	2129.8	15			

These Analyses show :

1. A significant treatment effect.

2. An error variance that tends to decrease.

3. The F ratio in the 3rd and 5th analyses of Trial I and II respectively is considerably greater than one, and though not significant at the 5% level, is an indication that there may be real residual effects, i.e. the response to any injection is affected by the size of any injection preceding it. This point is illustrated by a comparison of the treatment means in the second and third analyses of trial I and the 4th and 5th of Trial II.

Trial I

Treatment means	2nd response	14.0	37.5	50.3	71.5
" "	3rd "	29.3	26.5	18.3	16.0

Trial II

Treatment means	4th response	20.5	42.7	74.2	100.7
" "	5th "	31.5	42.30	26.8	24

The relation in Trial I gives a negative correlation coefficient of - 0.943, which is almost significant at the 5% level, and therefore suggests the presence of residual effects at the 2nd point. Trial II gives a non-significant correlation coefficient, though again a negative trend is observable. It may well be that any residual effect declines as the number of injections given increases.

In view of the rather inconclusive nature

of this data it was decided to repeat both trials using exactly the same procedure save that the four sows used would be of the same breed, the object being to reduce initial variation (compare 3 breeds used in Trial I, and 2 breeds in Trial II, necessitated at that stage by the availability of sows for this work).

Trial III

Square	Sows	Treatments
A B C D	Fay	½ - 1/4 - ½ units C
B A D C	Fatima	½ - ½ - ½ " D
C D B A	Ella	½ - 1 - ½ " A
D C A B	Felecia	½ - 2 - ½ " B

Fay	45	74	36	33	95	27	30	14	25	34	30	28
Fatima	60	137	55	43	86	34	43	39	41	40	12	15
Ella	53	20	47	35	32	32	51	113	32	38	90	41
Felecia	51	42	37	49	31	43	57	60	20	39	95	21

Trial IV

Square	Sows	Treatments
A B C D	Fay	½ - ½ - ½ - 1/4 - ½ units A
B A D C	Fatima	½ " B
C D B A	Ella	1 " C
D C A B	Felecia	2 " D

Fay	30	30	30	16	28	40	35	30	28	32	32	30	30	42	18	30	20	18	70	16
Fatima	44	42	36	34	23	35	29	30	18	23	24	17	16	37	14	25	20	18	32	14
Ella	40	30	30	55	30	40	36	30	80	20	32	30	28	27	24	30	29	30	15	27
Felecia	37	32	32	90	18	54	47	45	78	35	43	36	30	14	27	50	54	41	41	50

Analyses of Variance

Trial III

First Injection - All treatments same

Source	S.S.	df	M.S.	F.	Result
R.	413.75	3			
C.	498.25	3			
Tr.	70.25	3	23.42	1	N.S.
E.	225.0	6	37.5		
Ts.	1207.5	15			

Second Injection (Treatments 1/4, ½, 1 and 2 Units)

Source	S.S.	df	M.S.	F.	Result
R.	567.3	3			
C.	361.3	3			
T.	20271.80	3	6737.85	38.01	**
E.	1063.34	6	177.22		
Ts.	22263.80	15			

Third Injection (Treatments - residual effects only)

Source	S.S.	df	M.S.	F.	Result
R.	234.75	3			
C.	695.25	3			
T.	10.00	3	3	1	N.S.
E.	756.00	6	126		
Ts.	1695.50	9			

Trial IV

First Injection - (all treatments the same)

Source	S.S.	df.	M.S.	F.	Result
R.	495.75	3			
C.	225.75	3			
Tr.	178.25	3	59.42	1.95	N.S.
E.	183	6	30.5		
Ts.	1082.75	15			

Second Injection - (all treatments the same)

Source	S.S.	df.	M.S.	F.	Result
R.	449.2	3			
C.	160.2	3			
T.	407.20	3	135.73	2.143	N.S.
E.	379.85	6	63.31		
Ts.	1396.45	15			

Third Injection -(all treatments the same)

Source	S.S.	df.	M.S.	F.	Result
R.	331.75	3			
C.	176.25	3			
T.	200.25	3	66.75	2.45	N.S.
E.	163.50	6	27.25		
Ts.	871.75	15			

Fourth Injection (treatments 1/4, ½, 1 and 2 units)

Source	S.S.	df.	M.S.	F	Result
R.	1363.20	3			
C.	1105.70	3			
T.	6466.20	3	2155.40	29.63	**
E.	436.35	6	72.73		
Ts.	9371.45	15			

Fifth Injection (residual effects only)

Source	S.S.	df.	M.S.	F.	Result
R.	435.70	3			
C.	109.70	3			
T.	224.70	3	74.9	1.12	N.S.
E.	400.85	6	66.81		
Ts.	1170.95	15			

The outstanding feature of these analyses is the absence of significant residual effects.

In the light of this knowledge it appeared safe to assume the relation

$$y_2 - 15 = \sqrt{(y_1 - 15)(y_2 - 15)}$$ (Page 49)

would furnish a value of y_2 sufficiently accurate to be of use in the one point assay. A comparison of actual responses to predicted responses calculated by this technique confirmed the point - these figures have been taken from the 4 trials where 16 treatments of a half unit series were administered.

Response Observed	Response Calculated	Percentage Error
55	55	0%
39	39	0%
50	50	0%
27	28	4%
16	17	6%
37	39	6%
32	36	12%
65	55	18%
42	45	8%
32	33	3%
39	42	8%
30	31	3%
34	28	18%
28	31	19%
27	26	4%
41	45	8%
	Mean	7.3%

The Derivation of a Formulae

The object of biological standardization is to compare the potencies of two preparations, which when applied as stimuli to biological material produce responses of the same type, by means of the same or similar active constituents. If equally effective doses can be determined, and if it is known "a priori" that the preparations contain the same active principle and are therefore comparable, then this ratio, the relative potency, measures the value of

one preparation in terms of the other.

The linear relationship exhibited between response and log dose offered the possibility of assessing the dose producing any given response. While such a response regression is liable to shift from time to time, this shift probably represents an overall change in effective potency without any change in the sensitivity of the animals to variation in dose. In other words, the mean change in response per unit increase in dose may remain unaltered, even though the general level of response is not constant. If then a regression line for this standard preparation has been determined, a test preparation may be assayed relative to the standard by simultaneous experiment with one dose of the unknown and one of the standard.

It has already been shown that if y_s and y_u are the responses to the log doses x_s and x_u of the standard and test preparations, the general formulae relating them is

$$M = (x_s - x_u \quad - \frac{y_s - y_u}{b}$$

whence the relative potency is

$$R = \text{antilog } M.$$

If it is assumed that the standard and unknown are of equal potency, and that both are administered in the same doseage units, then $x_s = x_u$ and the equation reduces to

$$M = \frac{I\,(y_s - y_u)}{b}$$

where I is the log interval between doses used in determining b.

It follows that where possible the two doses should be chosen so as to be about equivalent, as the effect on the potency estimate of any change in the regression coefficient will thereby be minimized.

Applying this theory to the experimental data we have the following results.

The Value of the Regression Coefficient of Response on Log Dose.

The value of the regression coefficient of the response on log dose is given by the expression

$$b = \frac{\Sigma xy - \frac{1}{n}\left[\Sigma x\ \Sigma y\right]}{\Sigma x^2 - \frac{1}{n}(\Sigma x)^2}$$

Using the average of the 16 individual comparisons of response on log dose an average value of 24 was obtained as the value of b.

The formulae thus takes the form -

$$M = \frac{\log^2 (y_s - y_u)}{24}$$

which approximates to $\frac{y_s - y_u}{80.}$

A Comparison of Actual and Calculated Potencies

Again using the data provided by the four Latin square trials, and comparing the actual dose

given to that estimated from the response by the formula

$$M = \frac{y_s - y_u}{80}$$

the following results were obtained.

Trial I

Actual Response	Estimated Response	Actual Potency	Calculated Potency	Error as % of actual potency
y_u	y_s			
72	23	2.0	4.18	109%
78	27	4.0	4.33	8%
16	29	0.5	0.68	36%
65	55	1.0	1.33	33%
62	15	4.0	3.89	3%
36	20	2.0	1.58	22%
32	36	1.0	0.89	11%
19	63	0.5	0.28	44%
11	29	0.5	0.56	12%
37	39	1.0	0.94	6%
75	15	4.0	5.62	45%
56	42	2.0	1.49	26%
16	17	1.0	0.97	3%
10	15	0.5	0.85	30%
37	15	2.0	1.88	6%
71	30	4.0	3.29	15%

Trial II

Actual Response	Estimated Response	Actual Potency	Calculated Potency	Error as % of actual potency
y_u	y_s			
28	48	0.5	0.56	12%
90	24	4.0	6.68	67%
55	55	1.0	1.00	0%
60	20	2.0	3.16	58%
180	45	4.0	14.74	269%
25	31	0.5	0.83	66%
95	38	2.0	5.16	158%
39	39	1.0	1.00	0%
50	50	1.0	1.00	0%
60	26	2.0	2.66	33%
48	21	4.0	2.17	46%
17	34	0.5	0.61	22%
82	35	2.0	3.86	93%
27	28	1.0	0.97	3%
12	23	0.5	0.86	61%
85	20	4.0	6.49	62%

Trial III

Actual Response	Estimated Response	Actual Potency	Calculated Potency	Error as % of actual potency
y_u	y_s			
74	40	2.0	2.66	33%
137	58	4.0	9.7	143%
20	50	0.5	0.42	16%
42	45	1.0	0.92	8%
95	30	4.0	6.48	62%
86	38	2.0	3.98	99%
32	33	1.0	0.97	3%
31	46	0.5	0.64	28%
14	28	0.5	0.66	32%
39	42	1.0	0.82	8%
113	40	4.0	8.16	104%
60	35	2.0	2.05	8%
30	31	1.0	0.97	3%
12	20	0.5	0.79	58%
90	39	2.0	4.33	117%
95	27	4.0	7.08	77%

Trial IV

Actual Response	Estimated Response	Actual Potency	Calculated Potency	Error as % of actual potency
y_u	y_s			
16	29	0.5	0.68	36%
34	28	1.0	1.18	18%
55	30	2.0	2.05	3%
90	22	4.0	7.08	77%
28	31	1.0	0.92	8%
18	26	0.5	0.79	58%
80	24	4.0	5.01	25%
78	40	2.0	2.98	49%
42	22	2.0	1.78	11%
37	15	4.0	1.88	53%
27	26	1.0	1.03	3%
14	28	0.5	0.67	34%
70	17	4.0	4.59	15%
32	16	2.0	1.58	21%
15	28	0.5	0.68	36%
41	45	1.0	0.90	10%
			Average	41%

The Error Involved

The average error as a percentage of the actual potency computed over 64 assays is thus 41%, a result that may be considered satisfactory considering the refractory nature of the biological material employed.

Splitting this average error into the error for any limited dose range gives the following results.

Ratio y_s/y_u	Average Error
2 : 1	36%
1 : 1	7%
1 : 2	54%
1 : 4	67%

The low percentage error in the case of equal potencies of the test dose and the standard, illustrates well the point made earlier concerning an increased potential accuracy where the known and unknown are approximately equivalent, and this indicates that where this ideal is obtained, the assay is one of considerable accuracy.

S U M M A R Y

The practicability and accuracy of one point assays is established. The method used is entirely objective, being independant of a subjective matching of responses. The technique is simple and may be carried out rapidly, and where sufficient material is available to successively match the standard and unknown dose, a result of a high level of accuracy (± 10%) may be expected.

THE INFLUENCE OF MAMMARY PRESSURE UPON THE DURATION OF LET-DOWN

Prologue

Large day to day variations exist in the duration of the milk ejection response of the lactating sow to standard doses of Pitocin. Observations made in the course of early experimental studies upon milk let-down, indicated that the amount of milk in the gland, and the rate at which it was removed, influenced the duration of let-down. This observation suggested that mammary pressure might influence the period for which let-down occurs and so explain part at least of the variation encountered.

The end point of let-down in the cow is indeterminate, consequently it is difficult to estimate the period of response to any given dose of Pitocin and to elucidate factors affecting such a response. Both Peeters (1949) and Phillips (1952) have produced results concerning the behaviour of the gland in relation to mammary pressure, but neither workers' technique has enabled the problem of the influence of mammary pressure upon the period of let-down, and thus presumably the efficiency of the milking process in terms of milk obtained, to be studied. The sow assay technique enabled the problem to be tackled for the first time.

Experimental Method

The obvious manner in which to compare the

two factors, pressure and response, would be to measure both directly, keeping other variables as constant as possible, and to note the degree of correlation between the two. This simple procedure proved unpractical for a direct reading pressure tympanometer was unavailable, while teat cannulation with manometer coupling, was ruled out by the necessity for preserving the lactating properties of the sows. Thus an attempt was made to collect pertinent data using the indirect method of influencing the volume of liquid in the gland.

Removal of milk was undertaken in two ways, namely bulk removal with the aid of a large dose of pitocin, and gradual removal with a series of small doses. Mammary pressure was increased by the intraduct injection of large volumes of physiological saline, thus in all, 3 types of experiment were attempted.

This technique involves the assumption that any two glands of the one sow exhibit a parallel behaviour to varying doses of pitocin. This point was checked with the following results. (Fig.7.1)

<u>A Comparison of the Response of two teats of the one sow, when both are subjected to identical treatment.</u>

Two teats of the sow were selected at random. A series of intravenous pitocin injections was then administered, the two teats being milked at an approximately identical rate.

Results

	Dose (Units)	½	½	½	½	½
Sow I	Teat 1	65	55	50	55	55
	" 2	39	34	28	31	49
		½	½	½	1	½
Sow II	Teat 1	65	49	50	95	30
	" 2	60	40	45	91	30
		½	½	½	½	½
Sow III	Teat 1	35	32	32	31	22
	" 2	30	27	28	30	20
		½	½	½	2	½
Sow IV	Teat 1	55	35	20	60	20
	" 2	51	33	20	62	20
		½	1/4	½		
Sow V	Teat 1	53	20	47		
	" 2	43	20	30		
		½	1/4	½		
Sow VI	Teat 1	40	12	15		
	" 2	71	13	16		
		½	1	½		
Sow VII	Teat 1	40	95	44		
	" 2	38	90	41		
		½	1	½		
Sow VIII	Teat 1	43	86	34		
	" 2	37	80	30		

(Fig.7.1)

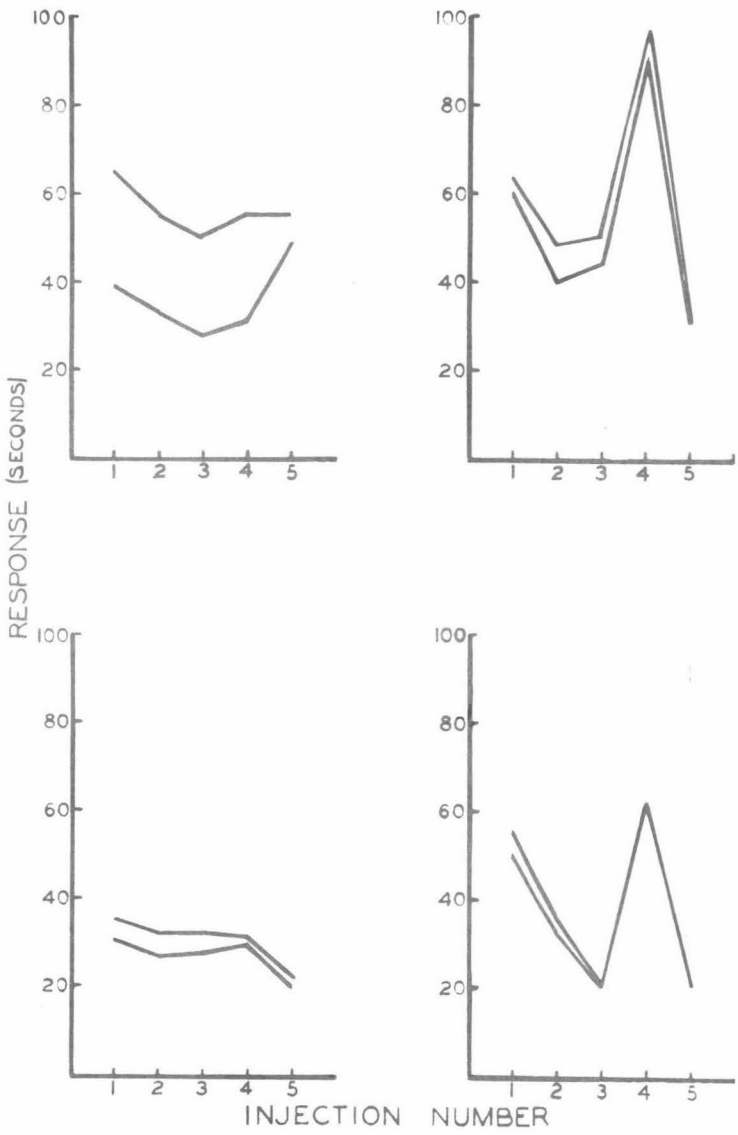


Fig.7.1a - A comparison of the response of two teats of the one sow when both are subjected to identical treatment.

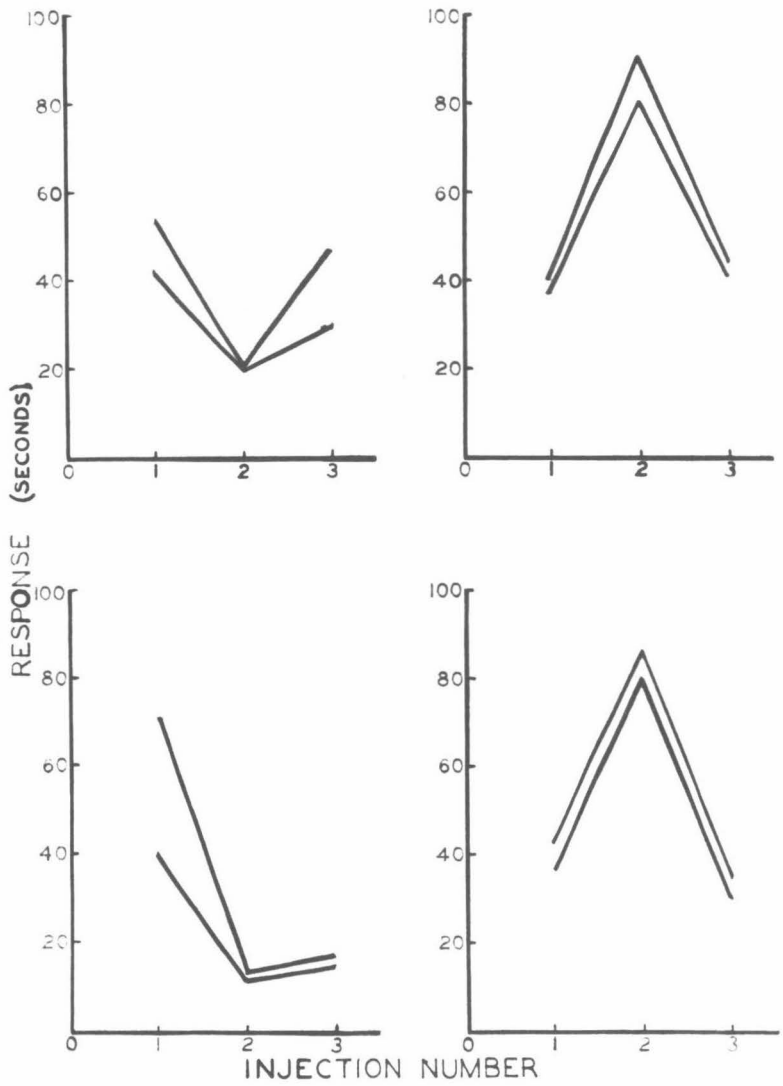


Fig.7.1b - A comparison of the response of two teats of the one sow when both are subjected to identical treatment.

It may be seen that a parallel pattern of behaviour existed between the two glands. It was concluded therefore that if a treatment applied to one gland only causes a disruption of this parallel behaviour, the disruption may be fairly attributed to the treatment applied.

A Comparison of the Responses to Standard Doses administered before and after the removal of a large sample of milk from one gland.

Two teats of a sow were selected such that they were adjacent and uniform in size and shape. A standard dose of pitocin was administered via the ear vein and the response of the two selected teats noted. A large dose (3 - 4 units) was then administered and the teat exhibiting the higher initial response milked as completely as possible. Two further half unit doses followed, the response of the two teats being compared in each case. Six sows were treated in this manner, repeats being carried out on four of them, such that 12 comparisons in all were obtained. These results are tabulated below. The first block represents the response of the two teats to the initial $\frac{1}{2}$ unit standard, the second and third blocks the responses of two further $\frac{1}{2}$ unit standards administered after a bulk sample of milk had been removed from one teat. Each row represents a separate trial.

I			II			III		
Un-treated	Treated	Sum	Un-treated	Treated	Sum	Un-treated	Treated	Sum
54	60	114	24	15	39	22	18	40
44	50	94	60	30	90	40	26	66
95	100	195	45	35	80	50	45	95
68	83	151	34	34	68	45	40	85
79	79	158	53	2	55	38	2	40
77	75	152	47	31	78	28	26	54
77	88	159	59	32	91	38	28	66
59	71	130	41	28	69	22	24	46
81	83	164	34	13	47	28	11	39
86	95	181	54	18	72	45	28	73
71	85	156	45	15	60	20	10	30
71	88	159	45	15	60	23	11	34
856	957	1813	541	268	809	399	269	668

(Fig. 7.2)

Analysis of Variance

A Comparison of the untreated and treated glands in each block.

I.

Source	S.S.	df.	M.S.	F.	5%	1%	Result
Rows	4133	11	375.7				
Treatment	325	1	325.0	10.18	4.84	9.65	**
Error	340	11	30.9				
Total	4798	23					

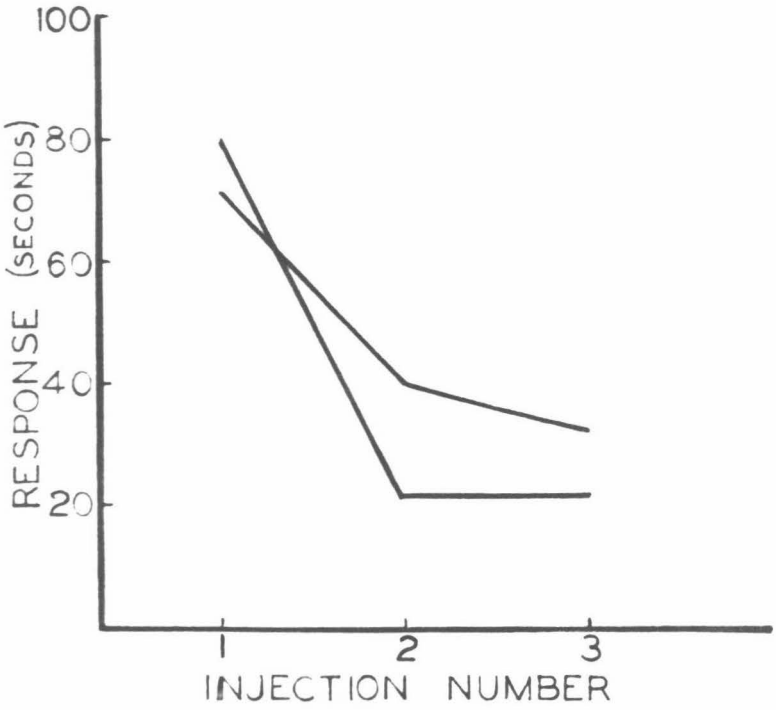


Fig.7.2 - A comparison of the responses to standard doses administered before and after the removal of a large sample of milk from one gland.

II

Source	S.S.	df.	M.S.	F.	5%	1%	Result
Rows	1424.5	11					
Treatment	3105.4	1	3105.40	31.6	4.84	9.65	* *
Error	1080.9	11					
Total	5611.0						

III

Source	S.S.	df.	M.S.	F.	5%	1%	Result
Rows	2451.4	11					
Treatment	704.23	1	704.23	10.46	4.84	9.65	* *
Error	740.8	11					
Total	3902.4	23					

The analysis of variance for Block I indicates that a significant difference in response existed between the 2 teats before any treatment was applied. In each case the teat exhibiting the greatest response was the one selected for treatment. The analysis of variance for Block II again indicates a significant difference between the two but in this case there has been a reversal in the high and low responses. This effect is carried through to the third response as indicated by the analysis of variance for block III.

In carrying out this experiment it was observed that the rate of milking, as well as the amount

of milk in the gland influenced the period of let-down. In the case of a teat that was milked lightly, the response was invariably longer than where a similar teat was milked firmly and rapidly. This observation is similar to one reported by Whittleston (1950) and Phillips (1952). Both these workers observed that in the case of an unmilked gland, the increase in milk pressure, brought about by the stimulation of let-down, remained at a fairly high level for a considerable period of time, provided the gland remained unmilked.

A Comparison of the Response of Consecutive Milking on one teat to the initial milking of other teats on the same sow.

A series of half unit standard doses were administered via the ear vein. The teat was selected as the control and milked on every injection; coincident with this milking another randomly chosen teat would be milked once only. This milk was steadily removed from one gland, and the response of this gland compared continually to previously unmilked glands. Eight comparisons were made in this manner.

Results

Fairy	Injection	1	2	3	4
	Teat 1	36	33	30	28
	2		45		
	3			48	
	4				50
	5	28	39	30	30
	6		37		
	7			45	
	8				46

Fiona	Injection	1	2	3	4	5
	Teat 1	35	45	25	23	20
	2	75				
	3		30			
	4			70		
	5				60	
	6					40

Pam	Injection	1	2	3
	Teat 1	111	62	48
	2	76		
	3		61	
	4			70

Fala	Injection	1	2	3	4	5	6	7
	Teat 1	60	50	35	25	25	22	25
	2	60						
	3		60					
	4			45				
	5				60			
	6					45		
	7						50	
	8							45

Gert	Injection	1	2	3	4	5	6	7
	Teat 1	42	45	28	26	14	20	20
	2	42						
	3		47					
	4			53				
	5				33			
	6					33		
	7						30	

Gail	Injection	1	2	3	4	5	6	7
	Teat 1	60	33	28	20	20	15	
	2	58						
	3		33					
	4			40				
	5				43			
	6					40		
	7						32	

Fairy	Injection	1	2	3	4	5	6	7	8	9
	Teat 1	70	52	46	43	33	26	23	16	14
	2	70								
	3		52							
	4			69						
	5				55					
	6					65				
	7						55			
	8							45		
	9								26	
	10									49

The summarized average response is thus -

	Injection								
	1	2	3	4	5	6	7	8	9
Continuously milked teat	55	45	34	28	22	21	23	16	14
Initially milking responses	64	46	55	50	45	42	45	26	49
Number of comparisons	6	8	8	7	5	4	3	1	1

(Fig.7.3)

It may be seen that the responses between glands appear dissimilar. The response curve for the continuously milked gland exhibits the usual curvilinear regression discussed previously, while that of the teats milked but once exhibit a straight line regression with considerably higher response values. As the difference between the glands being compared was the amount of milk in them, it would seem a safe conclusion to attribute the differences in response, in a large measure at least, to the amount of milk they contain.

It is of interest to speculate as to the cause of the negative slope of the regression line in the case of the initial milkings. Several hypotheses may be suggested.

(1) Insufficient data resulting in a chance regression varying from the expected zero value.

(2) The effect is indeed real and attributable to some physiological cause amongst which may be -

(a) The increasing fatigue of the myoepithelial cells of the mammary gland.

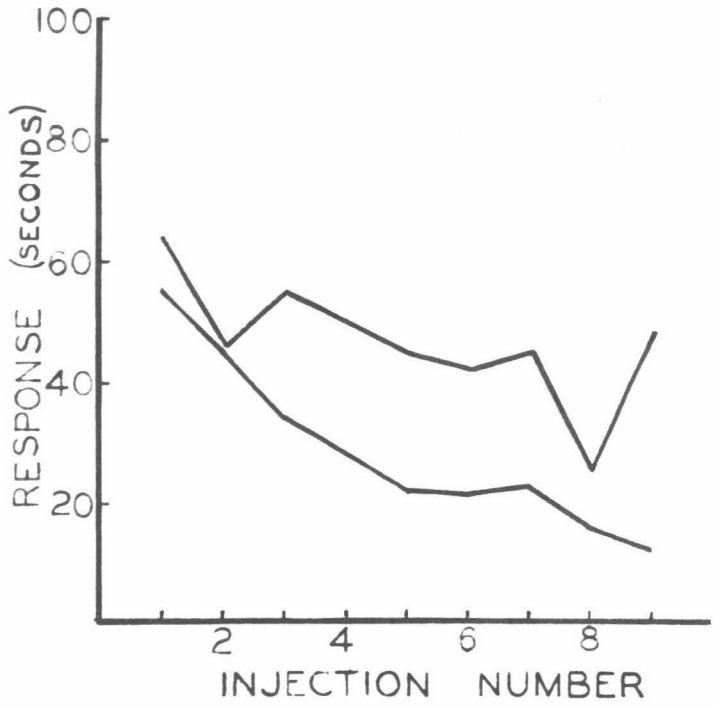


Fig.7.3 - A comparison of the response of consecutive milkings of one teat to the initial milking of other teats on the same sow.

(b) An increasing rate of pitocin inactivation, stimulated by previous injections.

(c) An increasing threshold value of blood pitocin concentration required by the myoepithelial cells for contraction.

(Note - It would be of little consequence to establish that the negative value of B is actually significant for it is known that milk production over the glands of a sow is not uniform. With 8 comparisons there is, even with randomized sampling, a fair chance of selecting the higher producing glands first, giving rise to an expected regression of the type encountered here. For the moment this observation must remain of hypothetical interest only.)

A Comparison of the response of two glands when the milk pressure of one is increased.

Two sows of those available at the piggery were culled after the Spring farrowing. Using these animals, an attempt was made to gather further information regarding the influence of mammary pressure upon the period of let-down by the process of removing milk from the gland, noting the decrease in the time of response to a standard, then filling the gland with physiological saline and again observing the response to a standard dose.

Two adjacent glands of uniform size and shape were selected and the response of both decreased

to a low value over a period of injections, both glands being milked uniformly and showing identical behaviour. 120 cc saline was then forced into one gland thereby increasing the response to a standard dose, the other gland remaining at its low level. The same treatment was then applied to this gland with identical results.

Sow I

Injection	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Dose (units)	½	½	3	½	½	3	½	½	½	3	½	½	½	3
Response - Gland 1	25	31	155	20	20	110	14	24	22	128	20	18	19	94
Response - Gland 2	30	33	155	14	20	102	14	14	14	52	10	16	19	111

Sow II

Injection	1	2	3	4	5	6	7	8
Dost (units)	½	3	½	½	½	½	½	½
Response - Gland 1	84	106	15	17	62	35	18	27
Response - Gland 2	81	-	37	32	22	20	53	51

In neither case has the response after gland infusion returned to its original level, in spite of the fact that approximately 70 cc of milk had been removed whilst 120 cc had been infused. This suggests that mammary pressure alone is not responsible for the declining response with continued injections. This finding lends reinforcement to the negative value of the regression of response on injection number over

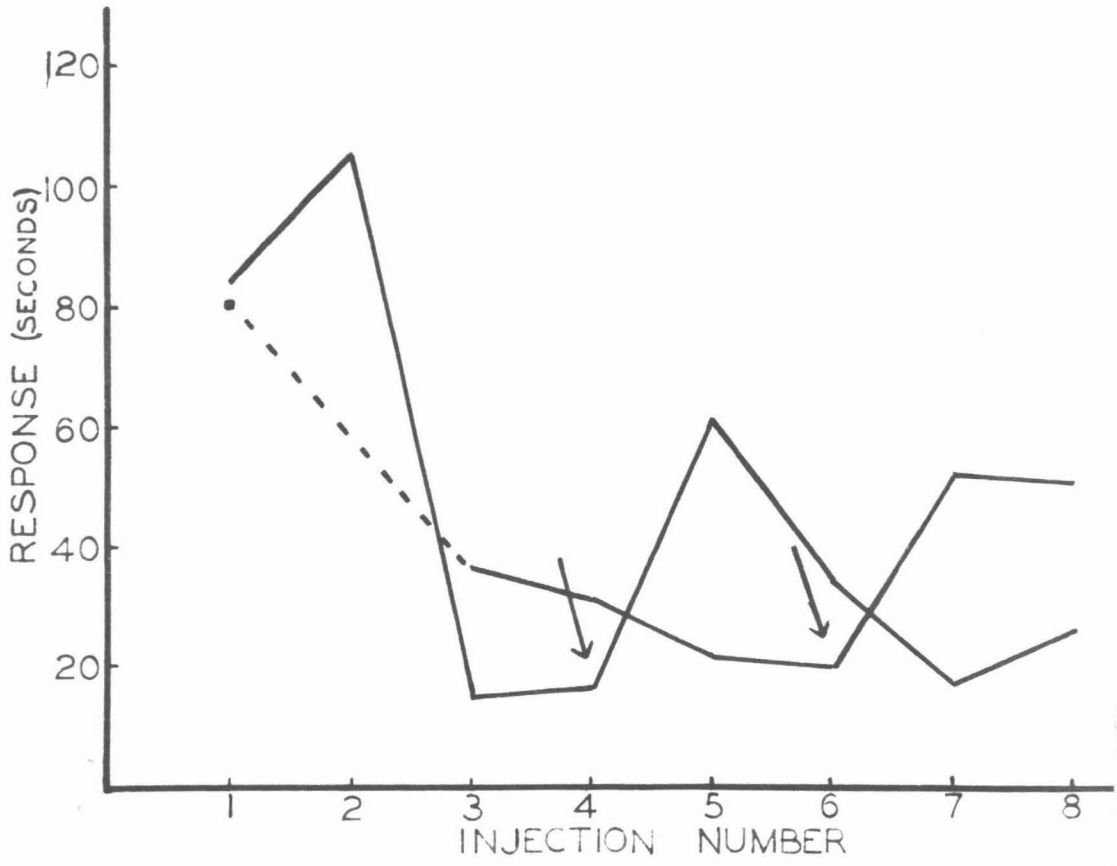


Fig.7.4a - A comparison of the response of two glands when the milk pressure in one is increased.

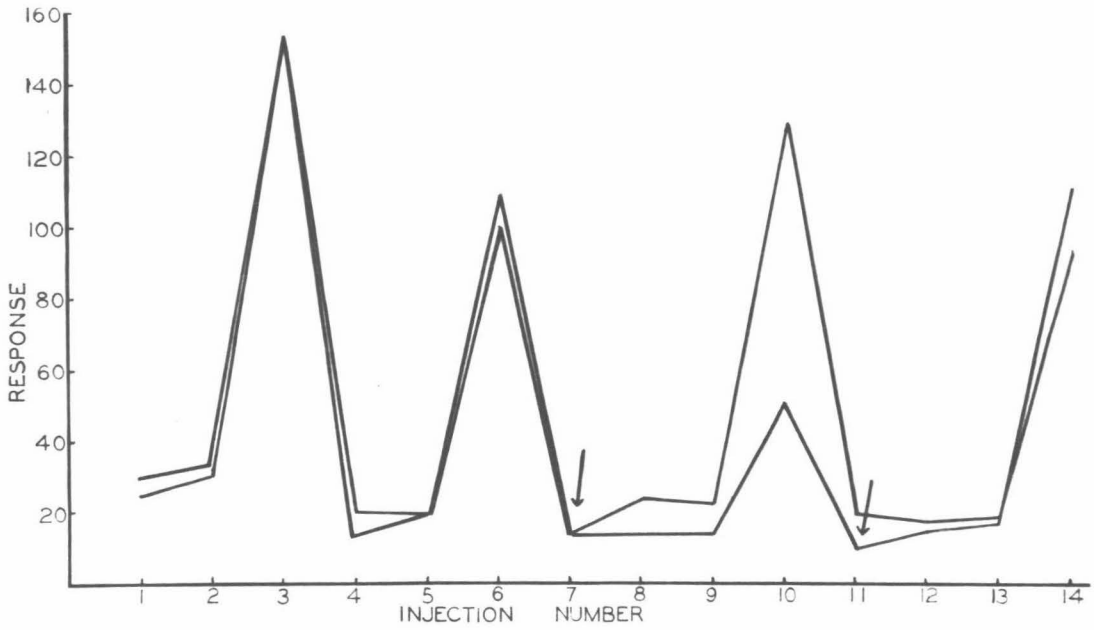


Fig.7.4b - A comparison of the response of two glands when the milk pressure in one is increased.

initially milked glands as discussed in Experiment II. Thus it would seem that some factor such as increasing threshold values, increasing enzymatic inactivation, or muscle fatigue, is involved in the phenomena of declining response with consecutive injections.

The Influence of Stage of Lactation upon the Response of the Gland.

The lactation period of a sow normally consists of a period of eight weeks, during which time the sow remains non-pregnant. Measurements of milk production made by Smith (1952) at Ruakura, show that milk production in the Berkshire breed is at a maximum during the fourth week of lactation, while data published by Hammond and Bonsma is in approximate agreement with this. In view of the fact that mammary pressure influences the period of let-down, it is conceivable that altering rates of milk production throughout lactation would give rise to a trend in response throughout lactation.

To test this hypothesis the standard responses of all Berkshires and Tamworths have been sorted into the period of lactation in which they were given. Where more than one injection has been given, only the first response has been taken.

Result - Berkshires

Week of Lactation	1	2	3	4	5	6	7	8
No. of Responses	1	3	5	16	13	9	8	3
Total	51	131	230	710	580	277	267	80
Mean	51	44	46	44	44	31	33	27

Tamworths.

Week of Lactation	1	2	3	4	5	6	7	8
No. of responses	-	-	4	9	6	5	11	18
Total	-	-	240	540	285	395	831	1268
Mean	-	-	60	60	48	79	75	70

While these results cannot be accepted as conclusive, the absence of any similar well defined trend in both the breeds makes it doubtful if stage of lactation exerts any appreciable influence on response. Whittleston (1952a) in a preliminary analysis of data reached a similar conclusion.

This result is of particular interest in relation to the involution of the gland. As intra-alveolar pressure appears to alter the response so markedly, this finding would suggest that throughout lactation the intra-alveolar pressure is at a reasonably constant level, yet the macroscopic appearance of the gland is such that one would conclude that its pressure varies considerably from the peak of lactation to involution. With the present lack of knowledge of the precise mechanism of mammary gland involution, the problem posed here can be merely left for future research

to clarify.

General Discussion

In the period following the classic experiment of Tgetgel, a number of workers have studied pressure changes in the mammary gland under the action of pitocin or natural stimulation. Their pressure measurements have invariably involved cannulating the gland and recording pressure changes by means of a manometer - a technique that, as Whittleston (1952a) pointed out, is merely qualitative, for the frictional forces involved produce a pressure drop and thus a recording that varies from the absolute value. The technique is however valid for measuring relative pressures. Of the recent papers published, two are particularly pertinent to the problem under consideration.

Peeters et al (1949) using their perfused mammary gland technique recorded a cistern pressure change of 10 to 24 cm. of milk after a 3 unit dose of pitocin. Filling the gland with milk until it recorded a cistern pressure of 35 cm. and repeating the 3 unit dose of pitocin, they recorded a pressure rise of 12 cm. From this work they concluded that pitocin increased the cistern pressure irrespective of an initial high or low pressure.

Phillips (1952) suggested that the let-down pressure of a cow was a relatively constant figure and independent of the amount of milk in the gland. He

reported that the time required for the milk pressure at milking to reach this maximum figure was considerable and variable, and increased as lactation advanced. In order to explain the non-variation in cistern pressure found over a major portion of the milk ejection curve even when an external pressure was applied, Phillips accepted and gave weight to the hypothesis of valve like opening and closing of the ducts supplying the cistern. He supposed a maximum equilibrium pressure between the alveolar system and cistern when the ducts were open; when closed the two were separate systems, the compensating mechanism, involving the milk storing capacity of the alveoli, being removed, so allowing pressure changes in the cistern. Phillips' results were explained in terms of a muscular mechanism squeezing the alveoli at the same time as the ducts opened. The muscular squeezing was postulated as being of constant tension producing a constant milk pressure. The "persistence time" i.e. the time of actual let-down was found to decrease with advancing lactation, while any upset of the cow resulted in a let-down of shorter persistence. When this occurred it was not infrequent for the rise in pressure due to let-down to have insufficient time to reach a normal maximum level before let-down ceased. Anomalous results whereby milk pressure remained below the normal level were interpreted in these terms.

Peeters and Phillips are thus at variance in

regard to the hypothesis of a maximum cistern ejection pressure, though both accept the hypothesis of a valve like opening of the ducts at the onset of let-down. Peeters has made use of extremes of pressure, Phillips only of the differences attributable to a.m. and p.m. milking. If both the cistern and alveoli system behave as relatively inelastic containers, as suggested, and a constant muscular tension is applied to the alveoli as Phillips postulates, it is hard to conceive that the alleged back flow of milk, caused by the application of an external pressure can indeed take place without being reflected in a pressure rise of both the alveoli system and the milk cistern, yet the problem remains of explaining the nonvariation of pressure throughout the major portion of the ejection curve and its variation thereafter. Indeed, if repeatable, this result would suggest that the mammary gland is not the inelastic system Phillips suggested it to be. The discrepancy between the results of Peeters and Phillips is possibly explainable in terms of a compromise, for Peeters, it may be argued, has worked with an "unphysiological extreme."

There can be little doubt that in the sow at least, the period for which milk is let-down is related to the amount of milk in the gland, and thereby presumably the mammary pressure. If the concept of a constant ejection pressure is valid, a contraction of less magnitude is required in the gland having an initial

high pressure, and conversely a contraction of greater magnitude is required in a gland with an initially low pressure. In other words, more work must be done by the myoepithelium in the latter case. As it has been shown that the period of contraction is dependent upon the doseage of pitocin, it follows that one might expect the muscles in a gland with an initially low pressure to contract for a shorter effective period, thus providing a possible explanation of the phenomena demonstrated in this chapter.

SUMMARY

1. The mammary glands of a sow are shown to exhibit parallel behaviour in their response to varying doses of pitocin.

2. The removal of a large sample of milk from a gland at one time is shown to decrease the response of a gland to further half-unit doses.

3. The removal of small amounts of milk from any one gland over a number of milkings depresses the response in comparison with that of a previously unmilked gland of the same sow.

4. Increasing the mammary pressure of a gland by infusion with saline has the effect of increasing the let-down time of the gland in response to the injection of pitocin.

5. There is some evidence that unknown factors such as muscle fatigue, increasing threshold values for contraction, or increasing rates of enzymatic inactivation, may exert an influence on the milk ejection response.

6. The mechanism of the manner in which mammary pressure influences the let-down period is postulated in terms of a constant ejection pressure.

THE INACTIVATION OF PITOCIN AND THE CESSATION OF MILK LET-DOWN.

Prologue

The period for which milk is available as a result of the stimulation of the pituitary by the handling of the udder, by the direct electrical stimulation of the neurohypophysis, or by intravenous injection of extracts of the posterior pituitary, is limited. The significance of this observation lies in the reports of those workers who have increased the yield of milk and fat as a result of the removal of residual milk by the use of pitocin over a number of consecutive milkings (Adams and Allen (1948), Smith (1949), Johanssen (1949)). These data suggest that a more complete physical milking may be capable of increasing production, and by corollary, the mechanism whereby the cessation of let-down is imposed may provide a limiting factor to the output of the mammary gland. This hypothesis is supported by observations commonly made by dairymen upon the necessity to "strip" certain cows if full production is to be obtained from them. An examination of factors involved in the cessation of let-down is warranted then, for if the hypothesis suggested above can be substantiated, we are measurably nearer our goal of increasing both the yield and efficiency of the production of milk and butterfat.

It has been demonstrated that the amount of milk in the gland is capable of influencing the period of let-down of milk, however this can hardly be

regarded as the final answer as to why let-down ceases after any one stimulation, yet general pharmacological evidence is such that it is unlikely that large amounts of the pituitary secretion accumulate in an active state outside of the pituitary gland. Thus it would appear likely that enzymatic inactivations are involved in the final destruction of the milk ejecting factor.

The Pitocinase Activity of Blood

A considerable amount of literature is available indicating that human pregnancy blood has the power of rapidly inactivating pitocin.

In 1930, Von Fekete reported that human pregnancy serum inactivated the posterior pituitary hormone, while in 1932 he showed that the oxytocic effect was reduced when the incubated mixture was tested on pregnant women as well as when it was assyed on the isolated uterus of a guinea pig. In 1935, Schochoert and Lambillan showed that pregnancy serum was also antagonistic to the vaso pressor activity of posterior pituitary extracts, and that normal pregnant women were relatively insensitive to the blood pressure raising properties of this hormone. Dieckmann and Michel made this observation independently at the same time.

Weile et al (1941) studied the inactivating power of pregnancy blood upon both the oxytocic and pressor activity. They observed the presence of an enzyme in the blood at the second month of pregnancy, and claimed that the concentration remained at a

fairly constant level from the third to the eighth month, a maximum being reached at parturition. They could no longer demonstrate its presence one month after parturition, nor could they detect it in the serum of non-pregnant women or in the blood of the foetal cord. Traces of the enzyme were, however, found in all urine specimens and in colostrum. The optimum pH for activity of the enzyme was found to be between 6.5 and 7.5, while the enzyme was thermolabile and easily oxidized. After oxidation it could be reactivated with cysteine or glutathione. No inhibiting substances could be found.

Page (1946) carried out further detailed studies on this enzyme, naming it pitocinase. He believed "pitocin" to be a large molecule consisting of a polypeptide containing at least 5 amino acids, having an active sulfdryl group and a molecular weight somewhere between 600 and 2000. He attributed the enzymatic inactivation of pitocin to the breaking of one of the peptide linkages, the substance so losing its pharmacological properties. Page found that the molecule was readily oxidized, a portion, but not all of the lost activity being restored by treatment with cysteine. Measurements of pitocinase in blood serum were carried out by inactivating pitocin under standard conditions. From the 4th to the 38th week after conception, he observed a thousand-fold increase in plasma pitocinase concentration, this high level being maintained until after parturition. The

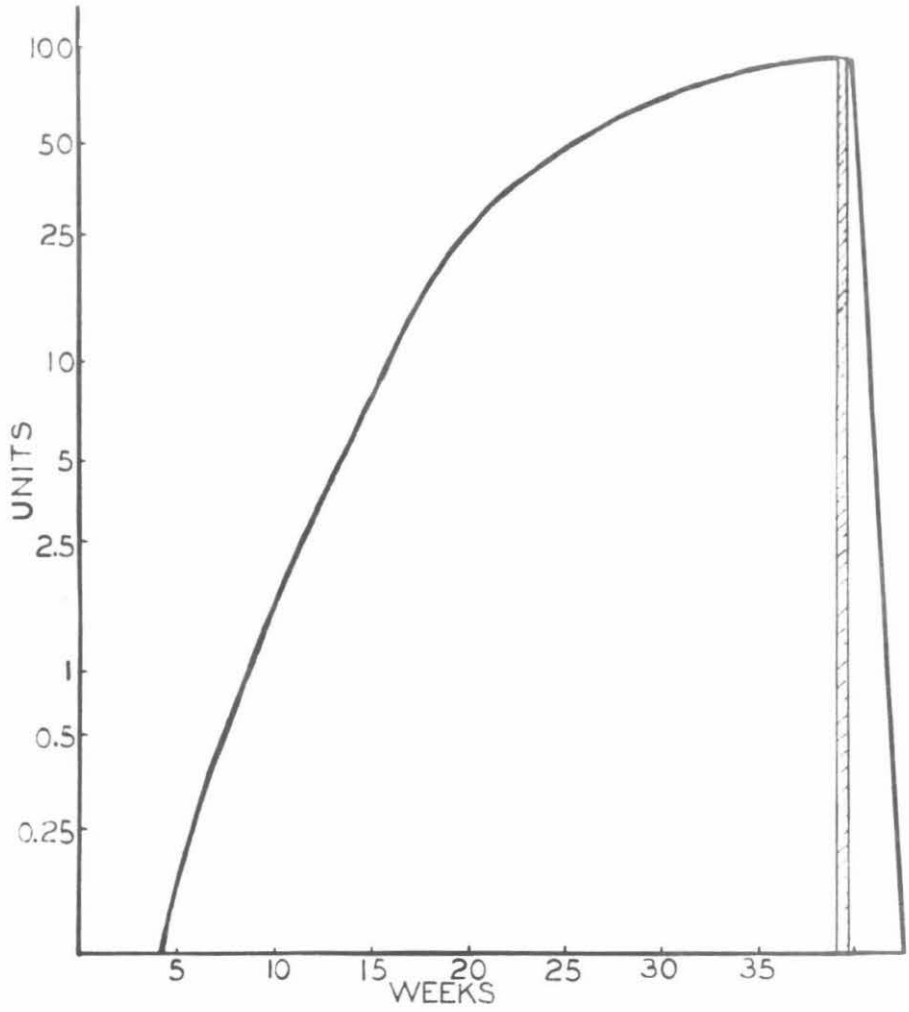


Fig.8.1 - Pitocinase concentration in the blood vascular system of pregnant humans as a function of stage of pregnancy. (From Page)

enzyme then decreased logarithmetically at the rate of 25% per day till absent within 4 weeks. (Fig.8.1) Page was unable to demonstrate the presence of pitocinase in the plasma of a rabbit, guinea pig or rat during their respective pregnancies.

A common observation made by dairymen is that as lactation proceeds the initiation and maintenance of let-down becomes progressively more difficult. As advancing lactation in dairy cattle is usually associated with advancing pregnancy, it is conceivable to think of higher levels of pitocinase circulating in the blood vascular system of such animals thus decreasing the efficiency of the natural secretion of the let-down factor (Whittleston, 1949).

Petersen and Ludwick (1942) and Peeters (1949) have presented data which may be interpreted as questioning the deproteinase activity of whole blood from normal lactating cows. Both workers, using perfused mammary glands found that the milk ejecting activity of blood from a cow stimulated to let-down retained its activity for 30 or more minutes. It is of interest to note that Peeters recorded milk ejection using normal unstimulated cows blood as the perfusion fluid, an ejection which was possibly brought about by the liberation of histamine within the blood itself (Peeters (1952), Code (1952)).

Jones and Schlapp (1936) and Heller and

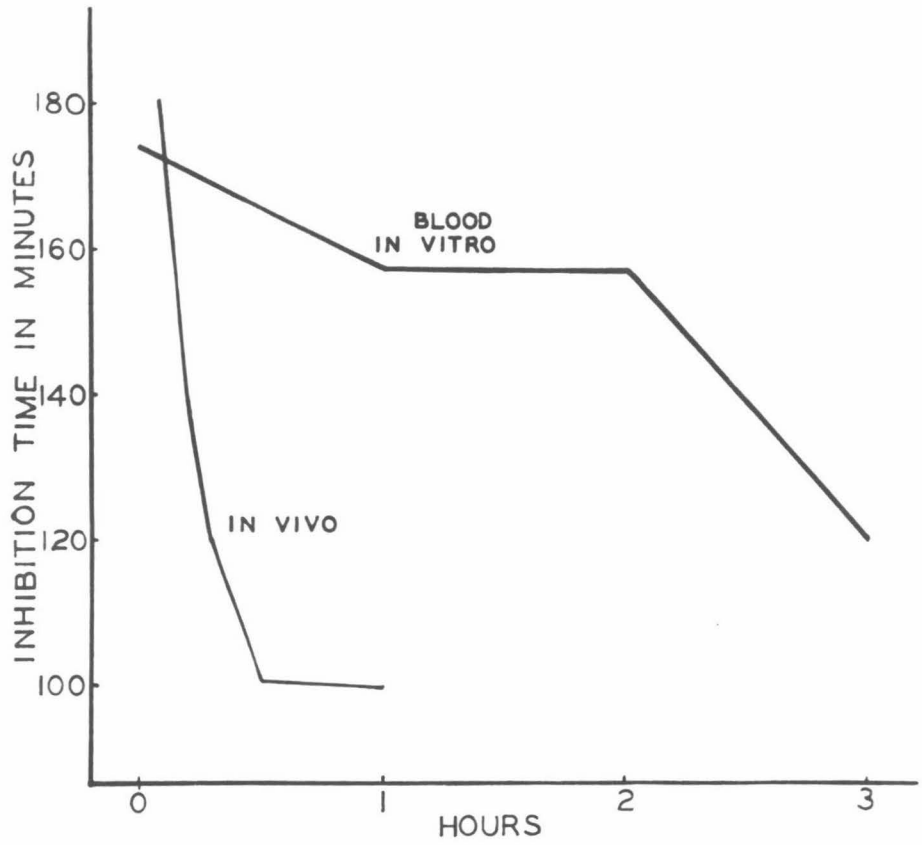


Fig.8.2 - In Vitro - Destruction of the antidiuretic hormone in the defibrinated blood of the rabbit. Concentration 50 m.u./cc.
In Vivo - Disappearance of antidiuretic activity from rabbits' blood after intravenous injection of 4 units per kg. of pituitrin. (From Heller and Urban)

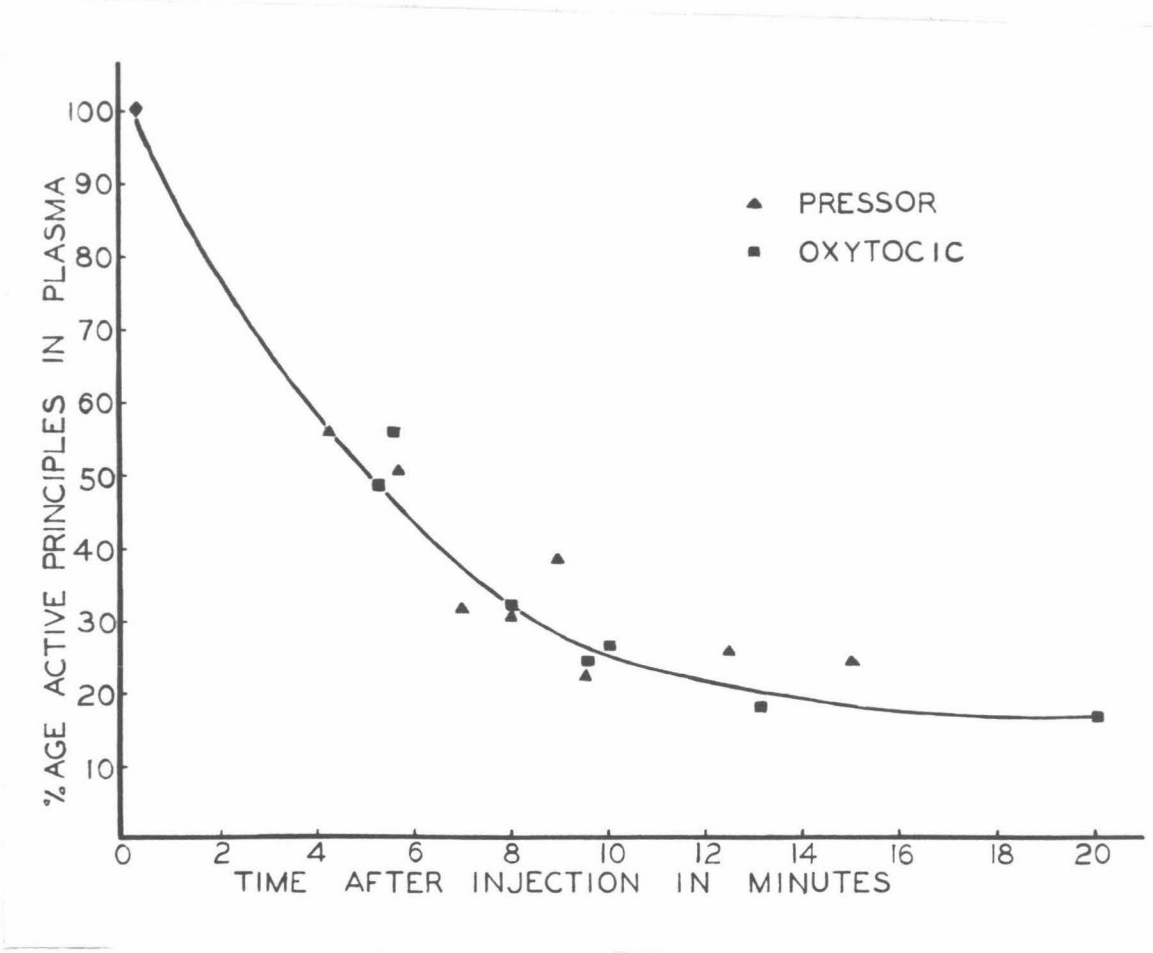


Fig.8.3 - A comparison of the rate of inactivation of the pressor and oxytocic fractions of posterior pituitary extracts administered to the circulating plasma of a decapitated cat. (From Jones and Schlapp)

Urban (1935) showed that there was no appreciable inactivation of the oxytocic, pressor and antidiuretic substances when posterior pituitary extracts were incubated with plasma. Using whole blood they recorded a slightly increased rate of inactivation in comparison to the plasma levels, but still at a non-significant rate. However they were able to demonstrate the inactivation of the pressor and oxytocic hormones in the decapitated cat, and the destruction of the antidiuretic principle in rabbit's blood within 10 minutes. Using an "in vitro" technique, they identified the liver and kidney as the source of inactivation. (Fig.8.2, Fig.8.3)

The Role of Excretion

In 1909, Dale was able to show that at least a portion of the pressor principle left the blood stream in an active state, for the urine of his test animals acquired a pressor response after the injection of pituitrin. Jones and Schlapp (1935) reinvestigated Dale's conclusions on a quantitative basis. They found oxytocic assays of the urine were impractical, for their urine samples exhibited an inherent oxtocic activity. This problem did not arise with pressor activity, consequently they were able to demonstrate that 28% of the pressor activity appeared in the urine (an average of 9 experiments), the urine itself having no inactivating power. Likewise Heller and Urban were able to demonstrate an excretion of the antidiuretic

principle, a result confirmed by Harris (1948). More recently Andersoon and Larssen (1952) have been able to demonstrate the excretion of an intidiuretic substance following milking in a lactating dairy cow. They concluded the milk ejecting factor and anti-diuretic principle were released simultaneously from the pituitary.

Experimental Methods

In order to clarify the rather confused picture presented by the literature, experiments were undertaken with the following four objects in view :

1. To learn the techniques associated with oxytocic assays, tissue extracts and enzymatic inactivations.
2. To determine the period of time during which the let-down hormone is operative in the cow.
3. To determine the possibility that fluctuating levels of pitocinase in the blood of pregnant cows might be responsible for the irregular let-down behaviour of cows in advanced lactation.
4. To determine any other potential source of inactivation within the animals body, and to make an introductory study of the nature of the inactivating process.

1. Oxytocic Assay

The method of Dale and Laidlaw (1912) which has been used until recently, employed the uterus of the virgin guinea pig. This method suffers from the disadvantages of variability in uterine sensitivity,

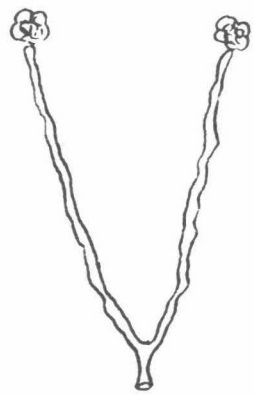
and the difficulty of obtaining suitable animals. Page (1946) used rat uteri with success, while Burn (1950) adopted the improved technique of Holton (1948). The technique used in this laboratory was essentially that used by Page.

Female rats culled for age and subject to estrogen treatment were the animals used. A doseage of 5 ug of estrone given over the three days preceding killing, served to sensitize the uterus to pitocin (Fig.8.4). The two horns of the one uterus were suspended in Tyrodes solution in a constant temperature bath (37°C) of 10 ml capacity. One end of each strip was fixed by a platinum hook to the bottom of the bath, the other end being attached to a length of thread, which in turn, was fastened to a lever so arranged that the shortening of the musclewas magnified in linear proportion on the kymograph drum. Air was bubbled through the Tyrodes solution, the bubbles passing between the two strips. (Fig.8.5 and 8.6)

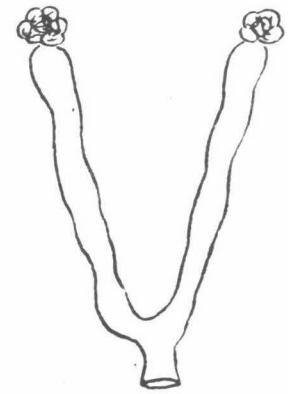
2. Deproteinase Estimations

(a) Blood Samples.

A blood sample of the order of 20 - 25 cc was taken from the animal concerned. This was immediately cooled, centrifuged and the resultant serum mixed with pitocin in the approximate ratio of 1 unit of pitocin to 100 cc of serum. Half this amount of mixture was immediately inactivated by adding one drop of dilute acetic acid (pitocin has its



UNTREATED



TREATED

Fig.8.4 - Representation of changes in the uterus of a rat as a result of estrogen treatment.

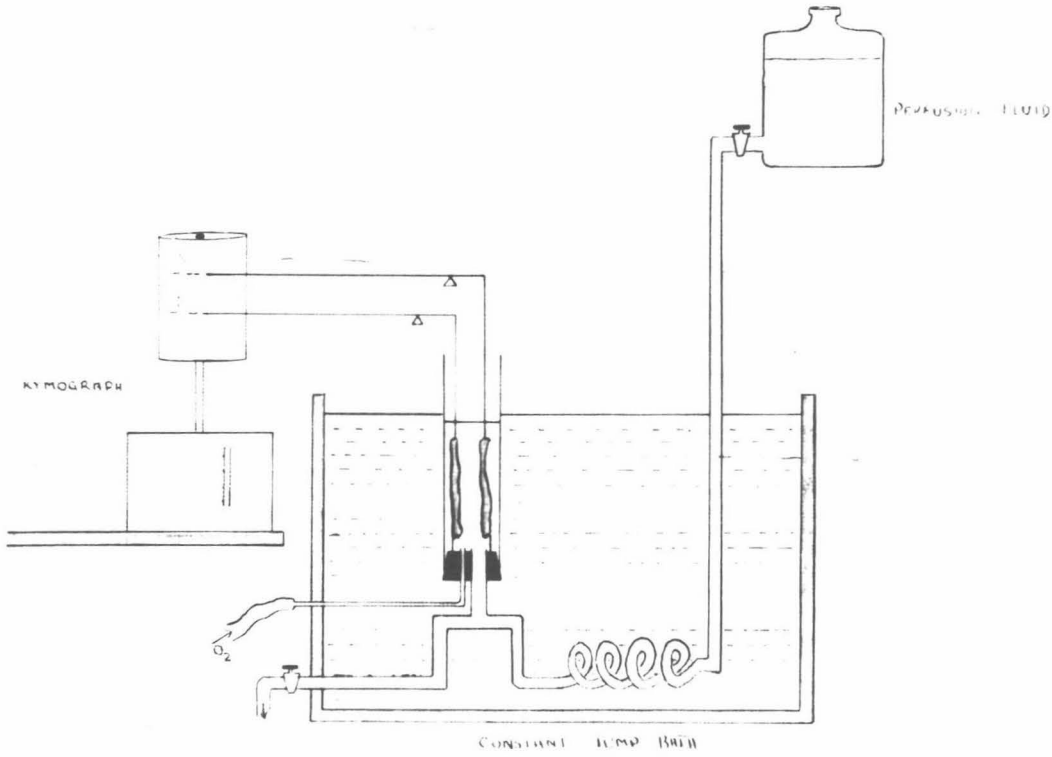


Fig.8.5 - Diagramatic layout of the apparatus used in oxytocic assays.

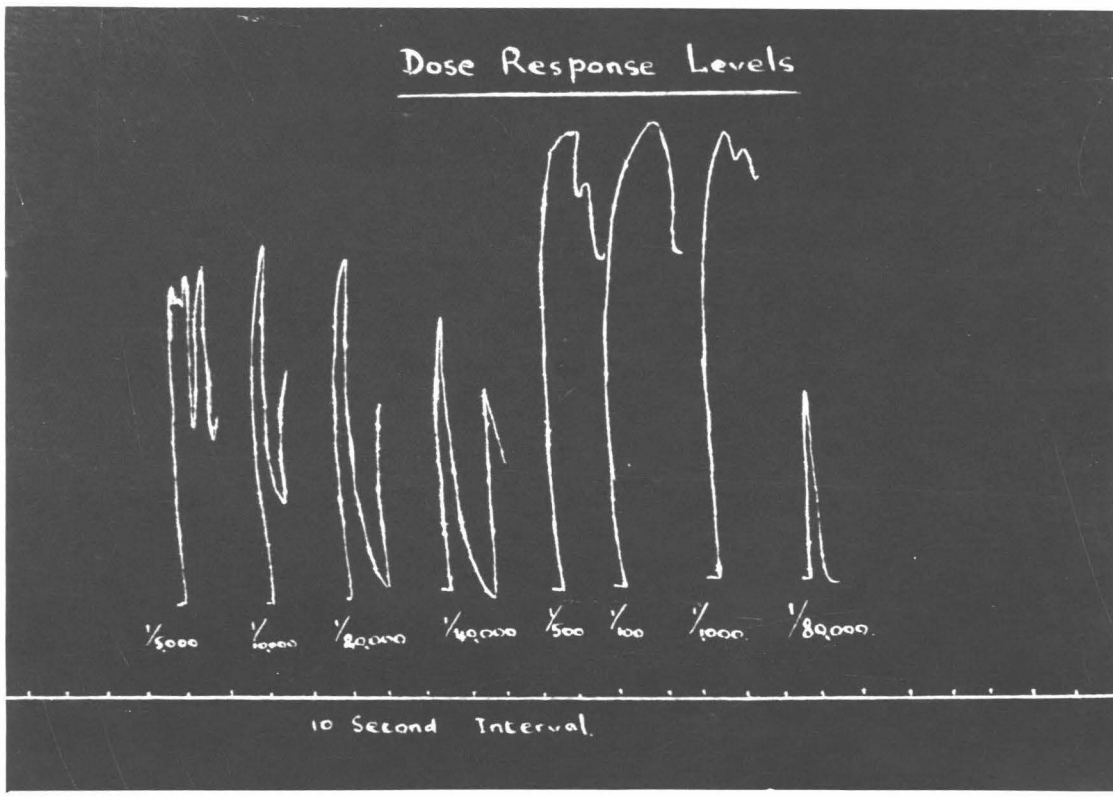


Fig.8.6 - A series of dose response recordings, illustrating the behaviour of the rat uterus under the conditions described.

greatest stability at pH 3 (Gaddum 1936)) and placing the tube in a boiling water bath for three minutes. The filtrate represented zero time or 100% substrate standard with which subsequent samples could be compared. The remainder of the mixture was incubated at 37°C and samples removed and tested at predetermined intervals. The percentage of pitocin remaining in each sample was estimated by a standard oxytocic assay on muscle strips.

Page (1946) reported that the destruction of pitocin by pregnancy plasma followed the kinetics of a first order reaction, for the log of the percentage of pitocin remaining plotted against time gave a straight line. Further, that the velocity of reaction was independent of the amount of pitocin used and varied only with the concentration of the enzyme in the incubation maxture. He concluded that the half time of pitocin destruction gave a measure of deproteinase activity. This estimate was used in these experiments.

(b) Tissue Samples.

The tissue to be studied was removed from the animal immediately after killing, and placed in Tyrodes solution. To prepare an extract, about 5 gram of tissue was ground with sand in a mortar and pestle, and a saline extract made of this mince. This was done by the addition of 20 ml of physiological

saline, the mixture well shaken and centrifuged at 3000 R.P.M. for 10 minutes. Deproteinase estimations were carried out as for blood samples from this point.

Results and Discussion

1. The "in vivo" inactivation of the oxytocic hormone.

At present there is no accurate method available for determining the concentration of the milk ejecting principle in the blood of an animal stimulated to let-down, consequently technical difficulties are encountered in studying the fate of these active substances. While it is theoretically possible to detect high levels of the oxytocic substance in the blood, the oxytocic contraction of a sensitized rat muscle due to such a factor is masked by the normal inherent oxytocic action of the blood alone. Thus attempts at following the oxytocic activity in consecutive blood samples taken from a cow after injection of 30 units of pitocin were only partially successful. The technique used involved taking a preliminary blood sample, injecting 30 units of pitocin and again taking samples at 2 minutes, 5 minutes, 10 minutes and 20 minutes. These samples were immediately placed in a refrigerator, two hours later they were centrifuged, the resulting plasma boiled with a drop of acetic acid and again centrifuged. The supernatant liquid was stored overnight in the refrigerator and tested for oxytocic activity the following day.

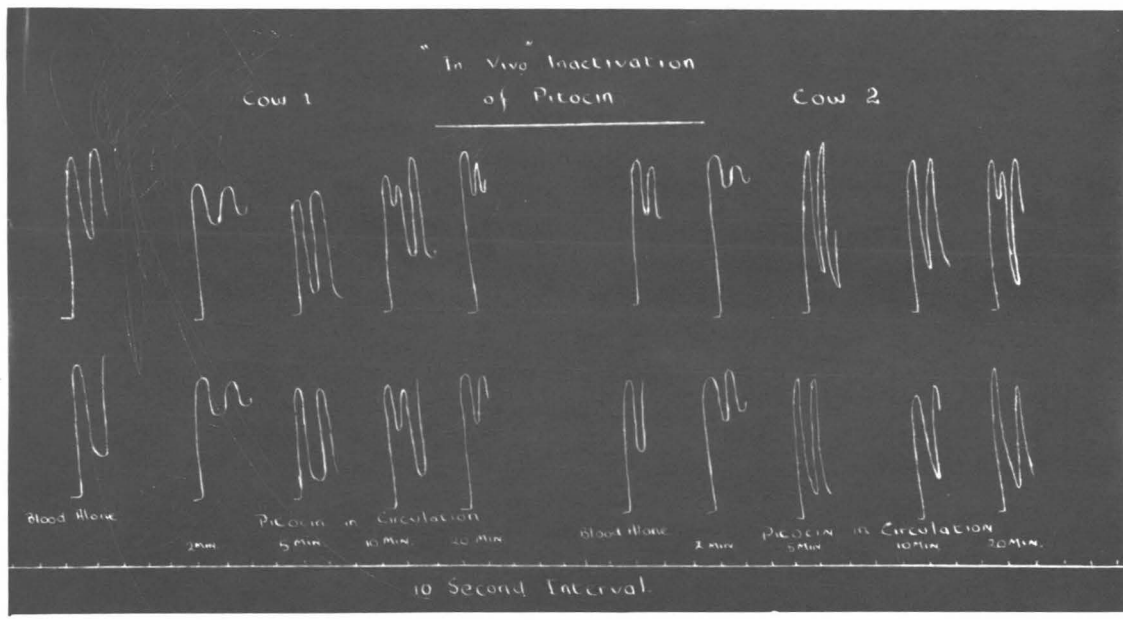


Fig.8.7 - The "in vivo" inactivation of the oxytocic factor within the circulatory system of a cow.

As the kymograph recording shows, (Fig.8.7) a maximum activity occurred at the two minute sampling in both animals, diminishing considerably at the 5 minute stage. This suggests an "in vivo" inactivation within this period, though the let-down of milk in both animals was observed to be still present at the ten minute sampling.

It is conceivable that considerable improvements might be made in this technique, while more animals would give a more decisive result. However, in view of the simultaneous "in vitro" inactivations observed, this problem was not pursued further for the purpose of this thesis.

2. The estimation of the pitocinase activity of cows blood.

Blood samples were taken from -

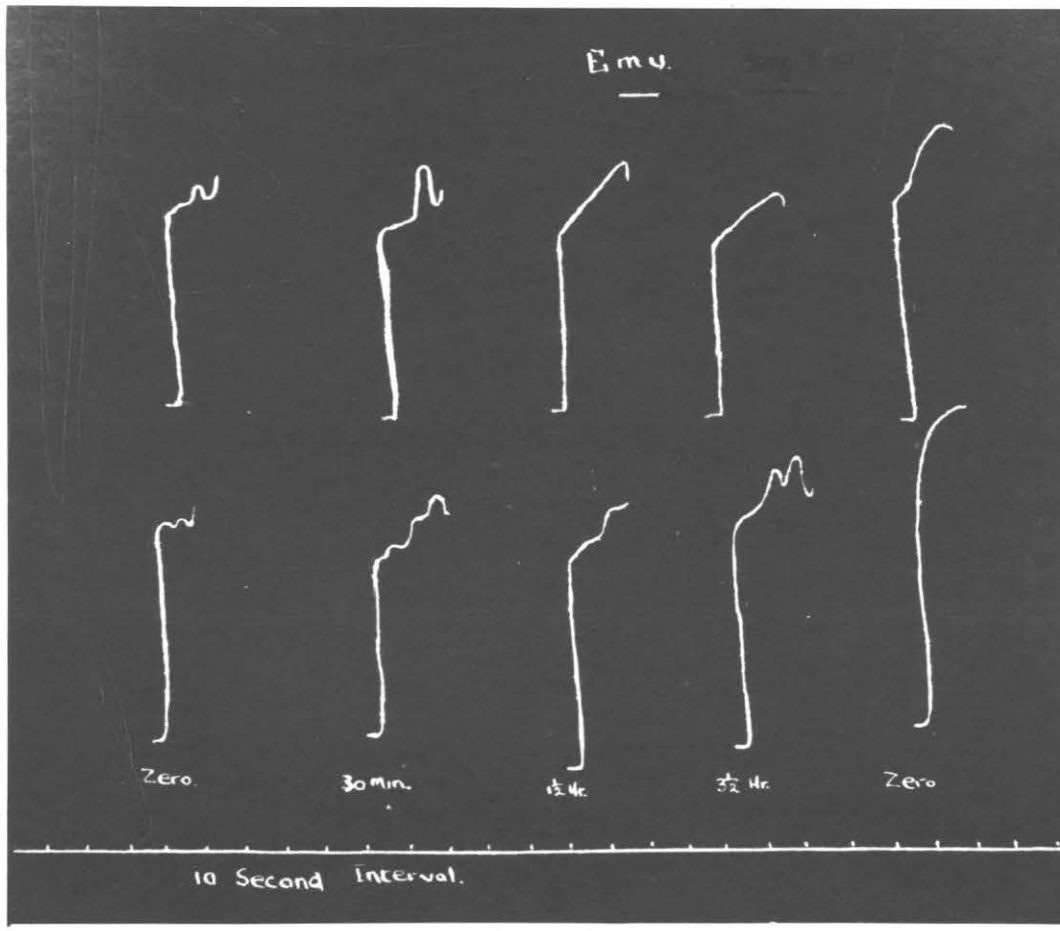
(1) 3 non-pregnant cows in declining lactation.

(2) A cow 7 months pregnant in declining lactation.

(3) 2 non-lactating cows approximately ten days prior to parturition.

There was no significant destruction of pitocin in any one of these 6 samples over an incubation period of two hours. (Fig.8.8)

The lack of deproteinase activity of non-pregnant blood is a result that one would expect from a review of the literature. The lack of deproteinase activity in the blood of the parturent animals is an interesting observation, for this result queries the validity of the hypothesis concerning the erratic



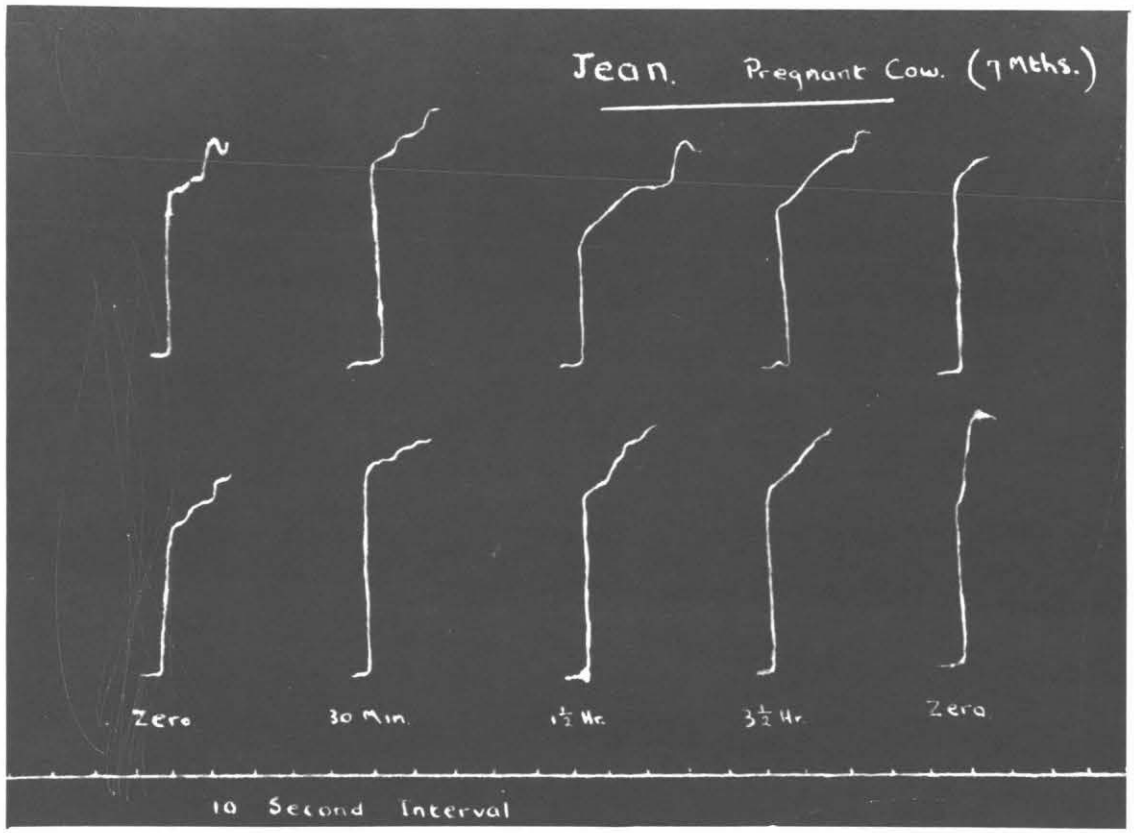
(a)

Fig.8.8 - The estimation of pitocinase activity of cows blood.

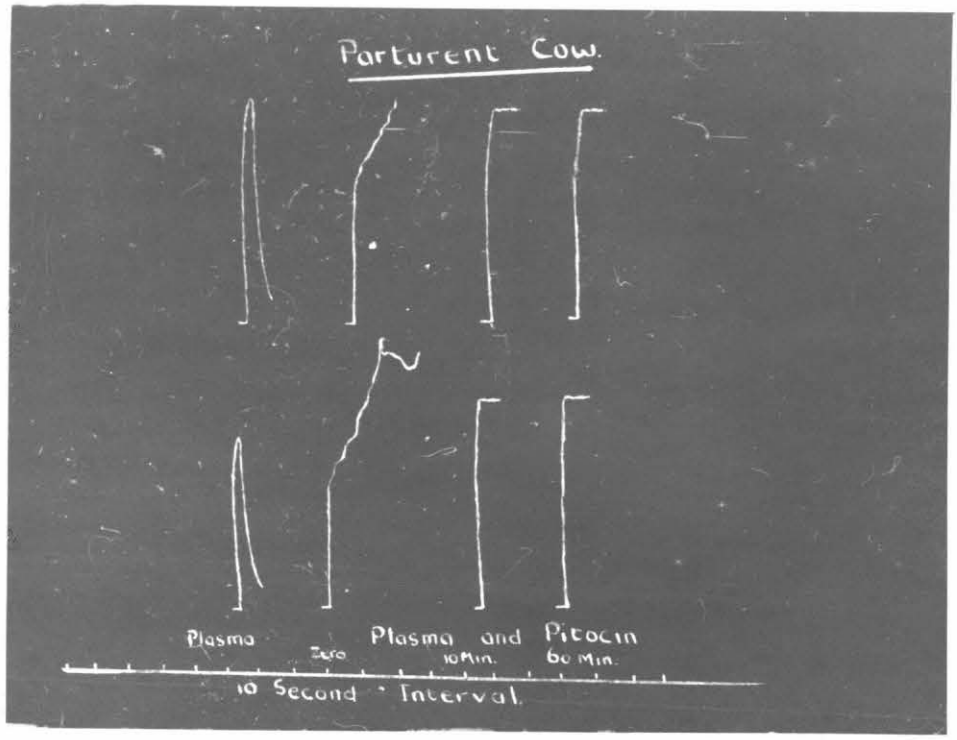
(a) - A non-pregnant animal at the end of lactation.

(b) - A pregnant cow at the end of lactation.

(c) - A parturent animal.


Jean. Pregnant Cow. (7 Mths.)
Zero
30 Min.
1½ Hr.
3½ Hr.
Zero
10 Second Interval

(b)


Parturent Cow.
Plasma
Zero
Plasma and Pitocin
10 Min.
60 Min.
10 Second Interval.

(c)

let-down behaviour of animals in advanced lactation. An alternative hypothesis for this observation may however now be postulated in terms of mammary pressure and let-down efficiency, for it has been shown in the work on the lactating sow that the amount of milk in the gland influences the period of let-down. As advancing lactation is associated with declining production and presumably declining mammary pressure, it appears justifiable to postulate that the decline in the efficiency of milk let-down is associated with declining production. Both these hypotheses assume the liberation of a consistent amount of the let-down hormone throughout lactation, an assumption open to criticism. Indeed it may well be that fluctuations and trends in the amount liberated following each stimulation, cause the milk ejection problems recorded by dairymen.

3. Tissue Extracts

Extracts were prepared from the heart, lungs, liver, kidney, spleen, and intestine of female rats, also the liver of a ewe slaughtered 3 days prior to extraction.

The liver and kidneys exhibited a consistent inactivating power, with a half time inactivation of approximately 2 minutes, likewise the ewe liver. The other organs exhibited considerably less activity, though appreciable inactivation was recorded after approximately 30 minutes. Using boiled extracts

there was no inactivation, a fact which suggests that the enzyme is a thermolabile substance. Liver extracts to which pitocin had been added and consequently destroyed showed no further activity after boiling, suggesting that the inactivation is irreversible and is a true destruction, rather than a tissue adsorption as postulated by Heller and Urban (1935). (Fig.8.9)

Page (1946) made the claim that the half-time inactivation of pitocin was proportional only to the concentration of pitocinase in his blood samples - provided physical factors were kept constant. Preliminary experiments in this laboratory indicated that this might not be the case with the liver extract - for a comparison of the times of inactivation for a one uhit, a four unit, and a 10 unit dose in equal amounts of extract, demonstrated that the respective times required to reach a concentration of 1 unit in 10,000 cc was such that the half times of inactivation must have been radically different (Fig.8.10). This result may be of significance in furnishing an explanation for the phenomena of tachyphylaxis, for one might explain the flattening out of the response with time curve, in terms of the antagonistic effects of decreasing let-down time due to the decrease in mammary pressure, and increasing let-down time due to a progressively decreasing rate of pitocin inactivation.

It is apparent then that enzymatic inactiv-

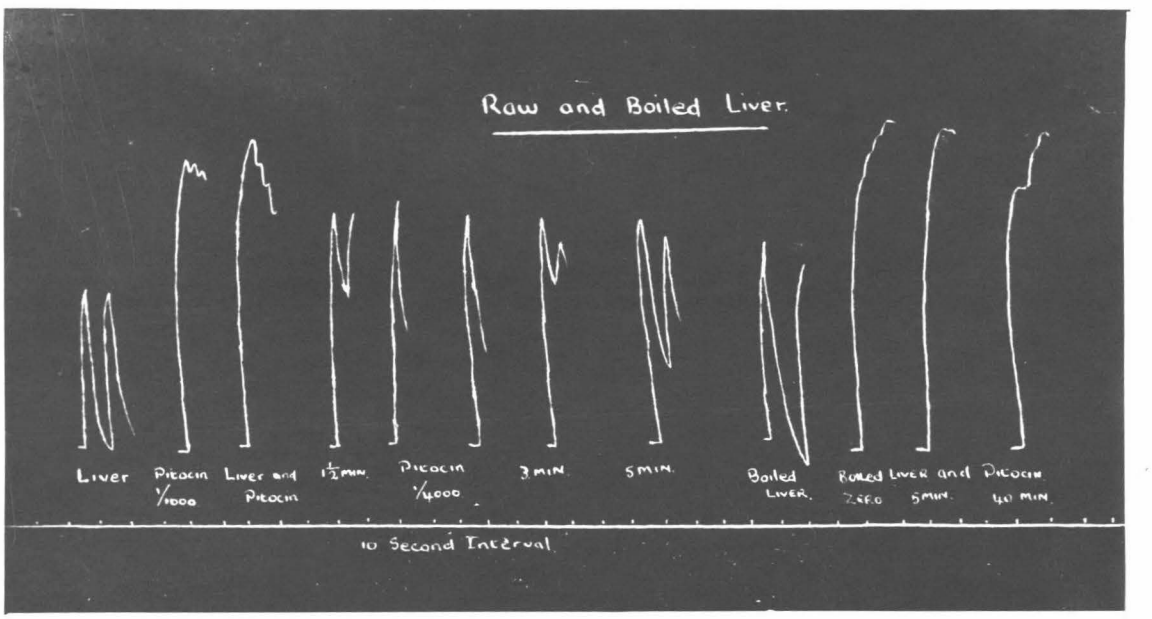


Fig.8.9a - The inactivating power of extracts of liver tissue both before and after boiling.

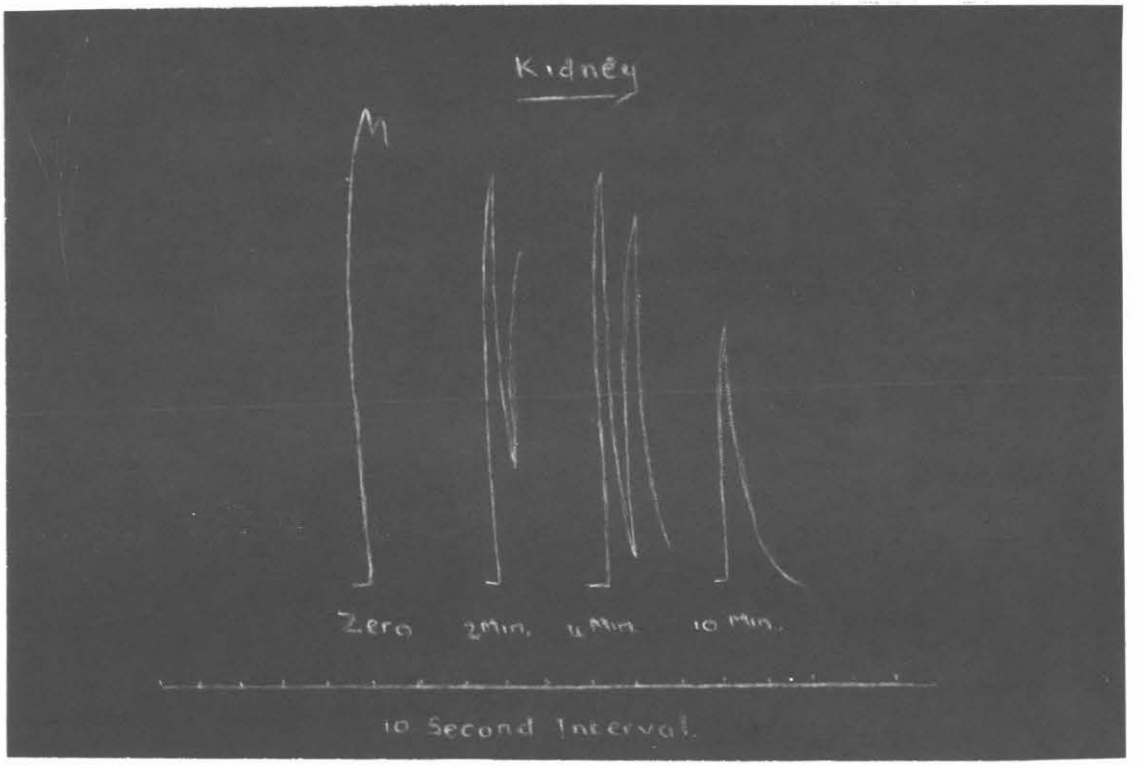


Fig.8.9b - The inactivating power of extracts of kidney tissue.

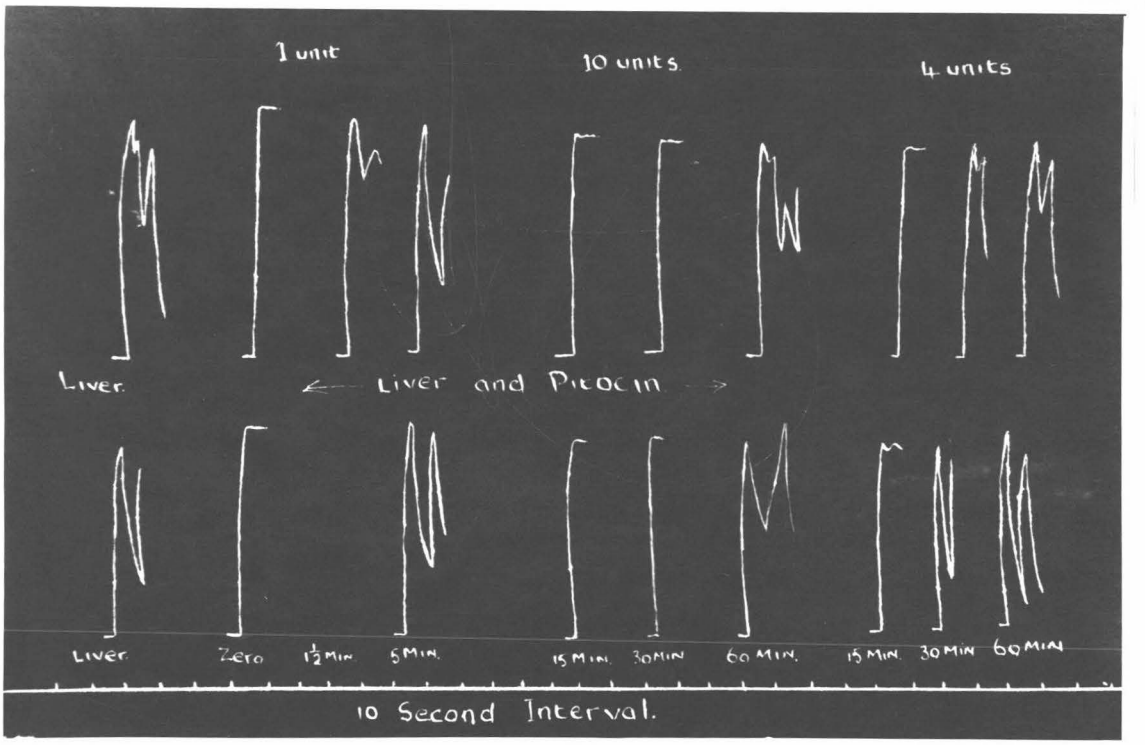


Fig.8.10 - The half-time of inactivation as a function of the amount of the otxtocic factor present.

ation of the factor causing milk ejection may provide a limitation as to the period for which is given down by the animal. However, in view of the fact that the let-down of milk does not cease simultaneously over all glands in the sow; the period of its availability being influenced by the pressure of milk in the gland, enzymatic inactivation can be regarded as only one of at least two factors bringing about the cessation of let-down. It appears probable that the enzyme systems constantly reduce the concentration of the let-down factor in the circulating blood, while the myoepithelial cells exhibit varying sensitivity, in accordance largely with mammary pressure.

SUMMARY

1. The problem of the site and the mechanism of the inactivation of the milk let-down factor is reviewed, for it is believed by the writer that the rapid cessation of milk let-down may constitute a limiting factor to the yield of a gland at any one milking.

2. Evidence is presented suggesting that the "in vivo" inactivation of pitocin in a lactating cow occupies a period of time of the order of 5 - 10 minutes.

3. Deproteinase estimations carried out on the blood of lactating non-pregnant cows, a lactating cow in advanced pregnancy and non-lactating parturent cows, revealed a complete absence of any factor in the blood capable of destroying pitocin.

4. Tissue extracts of the liver and kidney and to a lesser extent the heart, spleen and lungs, are capable of inactivating pitocin. The inactivating substance is thermolabile, produces a destruction irreversible by heating and appears to be modified in its rate of action by the amount of pitocin present.

5. The phenomena of tachyphylaxis may possibly be attributed to the compensatory mechanisms of the declining rate of inactivation of the let-down factor and the diminishing let-down response due to reduced mammary pressures. Such factors as myoepethelial fatigue and

an increasing threhold value for pitocin by the myoepethelial cells may also play a part.

6. It is concluded that the cessation of let-down is brought about by the operation of at least 2 factors -

(a) The level of mammary pressure within any one gland,

(b) The operation of an enzyme system attacking the functionally active groups of the factor causing let-down.

THE STUDY IN RETROSPECT

The genetic basis of milk production is believed to be a multifactorial one, whereby the additive and non-additive interactions of a large number of genes contribute the genic portion of the phenotypic variance exhibited by a herd of lactating dairy cows. Estimates of the heritability of milk production commonly range from 0.2 to 0.3, a figure which when used as the basis of calculating the genetic improvement possible in dairy cattle, results in an improvement estimated at approximately 2 lb of butterfat per year (Lush, 1949). There is however, a considerable amount of evidence suggesting that this hypothetical rate of improvement is not achieved in practice (N.Z. Dairy Board Reports, 1940-1950, Lush 1951). The cause of the discrepancy is the subject of conjecture and has given rise to speculation as to whether or not the observed discrepancy is actually an artefact. If the observation is correct, why therefore, it is asked, does the expected rate of gain still lack. Whatever the answer may be, it is apparent that improvement will be slow while selection is based on records of milk production, the heritability of which is so low.

There can be little doubt that ultimately milk production is monitored by the endocrine system, which in turn is governed in its activity by the products of the genes. Selection pressure in the

dairy animal is applied to the final phenotypic expression of the components, both genetic and environmental, of the physiological system of milk production. Thus, a complex chain of many interacting links exists between the formation of immediate gene products and such phenotypes. At each stage of this series environmental forces may produce some modifications, and thus the further the criteria of measurement used in selection is from the gene itself, the lower will be the heritability of the trait concerned. If then, an understanding of the endocrine basis of productive characters will permit the measurements to be made at earlier points in this series of physiological processes culminating in milk production, the use as criteria of factors with higher heritability than pertains to those criteria currently employed, might be developed with resultant improvement in the yields of dairy animals.

The process of milk production in the cow may be split into four components, each of which may provide a limiting factor to increased production in any one animal. Briefly stated these are -

1. The anatomical development of the mammary gland, together with a large secretory surface area.
2. The maintenance and continued activity of the cells of milk secretion within the gland itself.
3. The maintenance of an adequate flow of milk precursors to the mammary gland.

4. The successful removal of milk once formed.

This study has been made with the object of elucidating some aspects of the nature of the process of milk ejection, this being the first step in an effort to gain knowledge as to the importance of the removal of milk from the gland of the cow as one of the limiting factors in milk production. The next step will be to assess the importance of a "good let-down" in dairy cattle and to measure the heritability of this factor. In this manner it is considered the largely "hit and miss" techniques of present day animal breeding may gradually be improved to the stage where selective breeding is no longer a gamble, but becomes a means of securing within the one animal, optimum activity of those physiological processes contributing to maximum production.

SUMMARY

This manuscript presents a study of the physico-chemical relationships involved in the process of milk ejection.

A detailed review of the available literature concerning the neuro-endocrine relationships involved in the milk ejection reflex, as well as the innervation of the mammary gland and the mechanism of let-down, has been made. The concept that the posterior pituitary may provide a limiting factor to milk production has been examined and is substantiated; however the importance of this factor in what is regarded as a normal lactation is unknown. With a view to aiding the ultimate clarification of this latter point, a study has been made of milk ejection in the sow.

A technique has been developed whereby a large number of standard doses of pitocin may be administered consecutively and at standard intervals of time to a lactating sow. Using this procedure, and measuring milk ejection in terms of the period of time for which milk was actually "let down", it was found that individual sows exhibited a progressively declining response to such a series of standard size injections. The magnitude and rate of decline was found to be related to the breed of the animal used.

A linear relation has been shown to exist

between the log. of the dose administered and the response produced. This finding allowed suitable assay techniques for the milk ejecting activity of pitocin to be developed.

The amount of milk in the mammary glands of the sow has been found to influence the period of response to a standard dose of pitocin; however the influence of the stage of lactation of the sow appears to be without discernible effect. By making use of the standard rate uterine muscle oxytocic assay procedure, the ultimate destruction of the oxytocic factor by enzymatic inactivation has been demonstrated. It is believed that the dual operation of mammary pressure effects and enzymatic inactivation of the milk ejection factor bring about the cessation of let-down.

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APPENDIX - I

Milk Ejection Response

Note: All responses are for a half unit dose unless otherwise indicated by a figure in brackets next to the response. All recordings are in seconds. Responses for the experiments on milk pressure are reported in the text itself. Responses for the assays are in Appendix II.

A - LARGE WHITE SOWS

1. GLORIA - Farrowed 4th December.

Date of Treatment	Dec. 21	Dec. 28	Jan. 4	Jan. 24	Jan. 26	Jan. 28	Jan. 29
Response to 1st injection	34	38	31	60	58	60	40
2nd "	18	20	23	25	34	35	31
	21	15	19	25	34	28	28
	22	18	17	90(2)	25(1/4)	60(1)	27
	15			23	28	25	28
	11						

2. GLADIES - Farrowed 30th November.

Date of Treatment	Dec. 12	Dec. 29	Dec. 30	Jan. 2	Jan. 4	Jan. 24	Jan. 29
Response to 1st injection	40	65	53	37	51	20	90
2nd "	18	37	62(2)	36(1)	32	16	58
	10	26	8	16	27	14	39
	14(1)	34(2)			20		82(1)
		11			12		
					8		
					8		
					7		
					7		

B - TAMWORTH SOWS

1. PAM - Farrowed 8th December.

Date of Treatment	Dec. 27	Dec. 29	Dec. 30	Jan. 2	Jan. 4	Jan. 6	Jan. 7
Response to 1st Injection	40	65	53	37	51	68	90
2nd "	18	37	62(2)	36(1)	32	19(1/4)	104
	10	26	8	16	27	58	108
	14(1)	34(2)			20		41
		11			12		
					8		
					8		
					7		
					7		

Jan. 23		Jan. 24	Jan. 25	Jan. 28	Jan. 29
111	76	60	90	87	90
62	59	60	60	61	58
48	28	50	50	56	39
		28(1/4)	180(2)	50	82 (1)
		45	40	45	32

2. PEG - Farrowed 29th November.

Date of Treatment	Dec. 21	Dec. 28	Dec. 29	Dec. 30	Jan. 2	Jan. 4	Jan. 6
Response to 1st injection	89	48	65	31	-	45	72
2nd "	30	28	37	11(1/4)	26	75(2)	56(1)
	36	22	26	27	31	11	28
	27	19			13		
					12		
					12		
					13		

Jan. 20		Jan. 22		Jan. 25		Jan. 26	
79	79	77	75	44	50	60	60
-	-	-	-	-	-	45	45
53	-	47	-	60	-		
38	-	28	-	40	-		
				40	-		
				35	-		

3. FALA - Farrowed 12th November.

Date of Treatment	Dec. 7	Dec. 17	Dec. 19	Dec. 20	Dec. 27	Dec. 29	Dec. 30
Response to 1st injection	90	32	150	105	45	31	72
2nd "	35		83	58	26	23	72(1)
	29		58	33	26	13	14
			57	33	11	8(1/4)	
						8	
						8	

Jan. 2	Jan. 4	Jan. 6	Jan. 7	Jan. 9
40	43	82	128	
78(2)	16(1/4)	65	95	Assay
21	22	39	114	
		34	181(2)	
		37		
		15		

4. FANCY - Farrowed 3rd December.

Date of Treatment	Dec. 21	Dec. 28		Jan. 23	Jan. 24		Jan. 26	Jan. 28	Jan. 29
Response to 1st injection	75	26	-	Pressure work	55	-	84	85	50
2nd "	46	16	32		35	33	40	75	45
	28	10	21		20	20	42	35	40
	29	11	18		60	62	39	17(1/4)	85(2)
		9	15		20	20	36	33	15

5. FLICKA - Farrowed 16th November.

Date of Treatment	Dec. 7	Dec. 20	Dec. 27	Dec. 29
Response to 1st injection	60	71	38	23
2nd "	30	33	20	16
		16	16	8
			11	-

C - BERKSHIRE SOWS

1. ELLA - Farrowed 16th October

Date of Treatment	Nov. 25	Dec. 7
Response to 1st injection	18	20
2nd "		15
		14
		29(1)

ELLA - Farrowed 11th April

Date of Treatment	May 1		May 4		May 7	May 9		May 22		May 25	May 27
Response to 1st injection	43	53	35	30	51	38	40	40	40	40	30
2nd "	20	20(1/4)	32	27	113(2)	90	95(1)	36	39	30	29
	30	47	32	28	32	41	44	30	36	30	30
			31	30				80	95(2)	55	15(1/4)
			22	20				20	25	30	27
			18	12							
			14	10							
			12	10							

May 29
43
36
30
14(1/4)
27

2. FATIMA - Farrowed 7th October

Date of Treatment	Nov. 30
Response to 1st injection	27
2nd "	18
	15
	13

FATIMA - Farrowed 10th April

Date of Treatment	May 1		May 4		May 7		May 9		May 22		May 25	May 27
Response to 1st injn.	60		37	43	43	36	32	40	71	44	44	35	24
2nd "	137(2)		80	86(1)	39	42	32	12	13(1/4)	42	42	29	17
	55		30	34	41	33		15	16	31	36	30	16
										30	34	18(1/4)	37(2)
										20	23	23	14
										18	24		
										16	20		
										16	20		

May 29
25
20
18
32(1)
14

3. FRANCES - Farrowed 11th November

Date of Treatment	Dec. 7	Dec. 17	Dec. 19	Dec. 20	Dec. 27	Dec. 29	Jan. 7
Response to 1st injection	36	-	35	38	33	28	30
2nd "	21	28	94(1)		38	30	21
		25	24		35	24	
					11(1/4)	15	
					24	15	

4. FELECIA - Farrowed 20th October

Date of Treatment	Dec. 7	Dec. 12		Dec. 20
Response to 1st injection	35	32	18	28
2nd "	20	27	16	15
	17	20	16	18
	62(2)	19	17	11(1/4)
				15

FELECIA - Farrowed 24th April

Date of Treatment	May 1	May 4	May 7	May 9		May 22	May 25	May 27
Response to 1st injection	51	49	57	39	43	54	37	50
2nd "	42	31(1/4)	60	95	105(2)	47	32	54
	37	43	20	21	24	45	32	41
						78	90(2)	41
						35	18	50
								42
								34
								35

May 29
43
36
30
14(1/4)
27

5. FIONA - Farrowed 29th December.

Date of Treatment	Jan. 4	Jan. 20	Jan. 21	Jan. 24	Jan. 25	Jan. 28	Jan. 29
Response to 1st injection	25			64 39	40	60 65	39
2nd "	19			55 34	30	49 40	30
	12			50 28		50 45	25
	9			55 31		95 91(1)	48(2)
				60		30 30	18

6. FAY - Farrowed 8th April

Date of Treatment	May 1	May 4	May 7	May 9	May 22	May 25	May 27	May 29
Response to 1st injection	45	33	30	34	30	40	32	30
2nd "	74(1)	95(2)	14(1/4)	30	30	35	30	20
	36	27	25	28	30	30	30	18
					16(1/4)	28	42(1)	20
					28	32	18	16
						35		
						33		
						17		
						14		

APPENDIX - II

This appendix presents the responses recorded in carrying out the four point assays.

In each case the Latin square upon which the assay was based was of the form -

A	B	C	D
B	A	D	C
C	D	B	A
D	C	A	B

For each sow the 16 responses are recorded following this pattern. Beside each block of responses the relationship between the treatment and dose is indicated.

FALA

163	120	143	150	A = S_1 = $\frac{1}{2}$ unit of pitocin
78	72	125	113	B = U_1 = $\frac{1}{2}$ " " "
108	92	20	22	C = U_2 = 1 " " "
53	43	25	24	D = S_2 = 1 " " "

GLADYS

28	18	25	14	A = U_2 = 1 unit of pitocin
14	17	11	14	B = S_1 = $\frac{1}{2}$ " " "
13	11	11	12	C = S_2 = 1 " " "
8	8	7	7	D = U_1 = $\frac{1}{2}$ " " "

PEG

60	47	92	90	A = S_1	=	½	unit of pitocin
42	40	85	80	B = U_1	=	½	" " "
78	65	31	25	C = S_2	=	1	" " "
60	55	28	24	D = U_2	=	1	" " "

PAM

60	23	84	42	A = S_1	=	½	unit of pitocin
20	20	38	53	B = U_1	=	½	" " "
41	20	10	10	C = U_2	=	1	" " "
12	11	8	6	D = S_2	=	1	" " "

FALA

35	30	62	51	A = S_1	=	½	unit of pitocin
20	24	38	44	B = U_1	=	0.415	" " "
38	34	18	20	C = S_2	=	1	" " "
30	32	17	16	D = U_2	=	0.83	" " "

FIONA

35	31	75	45	A = S_1	=	0.72	unit of pitocin
16	25	32	50	B = U_1	=	0.5	" " "
30	19	11	9	C = S_2	=	1.44	" " "
13	12	6	8	D = U_2	=	1.0	" " "

A Six Point Assay

Sows - FIONA and PEG

Square

Sow 1.			Sow 2.				
A	B	C	D	E	F	F = U_1 = 1/4	unit of pitocin
B	C	F	A	D	E	B = U_2 = $\frac{1}{2}$	-do-
C	F	B	E	A	D	C = U_3 = 1	-do-
D	E	A	B	F	C	A = S_1 = 3/8	-do-
E	A	D	F	C	B	E = S_2 = 3/4	-do-
F	D	E	C	B	A	D = S_3 = $1\frac{1}{2}$	-do-
28	18	35	300	100	25		
16	34	11	27	98	33		
37	11	19	28	18	60		
42	20	15	86	31	115		
30	12	46	20	78	35		
8	46	15	60	20	17		