STUDIES ON THE PERFUSION OF BOVINE MAMMARY GLAND

by

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INTRODUCTION

The last three decades have seen unprecedented strides in our knowledge concerning the chemical reactions constituting the life processes of tissues and organs of the highly evolved forms of animal life, on the one hand, and on the other, those associated with the simple unicellular organisms like the bacteria, yeasts, etc. A fundamental similarity of all life processes emerged from these studies, which was illustrated so classically by the manner in which the studies of the mechanisms of alcoholic fermentation of yeasts and of muscle glycolysis complemented one another.

Most of our knowledge regarding the tissue metabolism have been obtained by the Warburg technique in which a thin slice the tissue under investigation is studied with respect to either, 1) the requirements for its metabolism, or 2) the characteristics and the factors affecting a single reaction, isolated from the whole reaction sequence. A wealth of valuable information has been obtained regarding the nature of the biochemical reactions occurring in the living cells, especially regarding the enzyme specificities, coenzyme requirements, specific inhibitors, etc.

The use of the tissue homogenates and more recently the reconstituted enzyme systems were further refinements of the tissue slice technique. These modifications were introduced for facilitating the study of a specific reaction in greater detail than was possible with the tissue slices. It is obvious that in these methods the emphasis is placed on the attempt at

understanding individual chemical reactions rather than on the overall metabolism of the tissue or the organ from which the tissue is obtained.

If, however, an organ, like the mammary gland, is under investigation with respect to its overall metabolism, the tissue slice technique or any of its modifications is not applicable, mainly because a product of the metabolism, as complex as milk, is involved. Milk although very complex in composition shows a remarkable constancy in composition under normally varying physiological conditions. It must be assumed, therefore, that all the biochemical processes concerned with milk secretion are interrelated and are taking place simultaneously. The isolation of one reaction or one set of reactions from the innumerable other reactions occurring in the mammary gland will have to await a more complete knowledge regarding the processes involved. The lactating bovine mammary gland is one of the most active organs of the body, synthesizing simultaneously relatively large amounts of distinct carbohydrate, fat and proteins, all unique in the animal kingdom. At the present state of knowledge it appears desirable to obtain more information regarding the precursors of some of these chemical constituents of milk.

The arteriovenous difference studies gave extremely valuable information regarding the metabolites of the gland. Glucose, beta-hydroxybutyric acid, acetic acid and amino acids are some of the metabolites shown to be utilized by the mammary gland in the intact cow. The arteriovenous differences in any substance show the loss of this substance during the passage of the blood

through the gland. The substances lost are evidently utilized by the gland for its metabolism. If, however, the gland could be isolated and maintained physiologically active by perfusion with blood, the fate of a suspected metabolite added to the blood could be studied. A passage of the blood through the gland ten times would accentuate the arteriovenous difference ten times also and consequently substances utilized in minute amounts could be estimated with greater accuracy. Secondly, the effect of heavy doses of individual substrates in the blood could be determined. Thirdly, the use of tracer compounds for the study of milk secretion uncomplicated by the presence of other organs would be possible.

Realizing the advantages in the use of the perfusion techniques physiologists have attempted the perfusion of organs since the middle of the last century. Practically all of the organs of the vertebrate body have been used, with mixed results. The general consensus of opinion has been that obtaining a rate of flow comparable to that of the organ in the body has so far been impossible.

It has been a matter of doubt whether an organ, even when supplied with adequate quantities of blood by artificial perfusion was functioning physiologically since a complete cessation of the blood supply to the organ occurs during the interval between the shedding of the blood and the beginning of the perfusion. Recent work (91) on cardiopulmunary surgery of dogs have been heartening. It appears that no irreparable

damage is done to any part of the body, with the exception of the brain, by completely witholding the blood supply for as long as forty minutes. From this, qualitatively normal metabolism can reasonably be expected by perfusion if the time lapse is less than forty minutes.

The first part of the study reported in this thesis was undertaken to establish conditions necessary for maintaining the isolated bovine mammary gland in as close a physiological state as possible, the criterion being the rate of blood flow. The second part of the study deals with the effect of the presence of acetic acid in the blood on the utilization of beta-hydroxybutyric acid by the mammary gland. This investigation provides an example of the type of studies that can be made by the use of the perfusion technique which would be difficult, if not impossible, in an intact cow.

Part I

Factors Affecting the Rate of Blood Flow through the Isolated Bovine Mammary Gland

A perfusion apparatus, in essence, consists of 1) a pump which supplies the organ under investigation with the perfusate, 2) an oxygenator, to restore the oxygen content of the blood depleted during the passage of the perfusate through the organ, 3) a reservoir from which the perfusate is pumped back into the organ and 4) a temperature controlling device.

That life might be maintained in any portion of the animal isolated from the rest of the body by artificial means, e.g. perfusion with blood was suggested in 1813 by La Gallois (48). It remained, however, for others to actually undertake such experiments. Kay (43), in 1828, found that irritability in a dying muscle could be maintained by perfusing it with blood. Similar findings that life could be prolonged in other organs by such artificial means were reported by other workers. Brown-Sequard (14) showed the necessity of the oxygenation of the perfusate. Fry and Gruber (25) attached the piston of a syringe supplying the artery of the organ to a motor-driven wheel and thus created a mechanical means for pusatile pressure. They also devised an arrangement by which the movements of a pendulum interrupted the flow of blood intermittently and thus created artificial pulsation. These early works represented extremely crude attempts to simulate natural conditions and the rate of flow of blood was far from that obtained through the organ <u>in vivo</u>. Oxygenation was effected by bubbling air or oxygen through the blood which caused considerable foaming and consequently embolism of the organ. In order to overcome this,

Jocobj (38) used the lung of another animal in which respiration was artificially maintained for the aeration of the blood. Since these mechanical methods of perfusion proved highly unsatisfactory, Martin (55), as far back as 1881, attempted the study of the activity of the heart by directing all of the blood from the aorta back into the right auricle. A live heart-lung preparation was thus effected in which the circulation of the blood was exduded from the rest of the body. Bainbridge and Evans (6), in 1914, substituted this living preparation for the artificial perfusion machine. The organ to be perfused received its blood directly from the aorta of the preparation. The blood issuing out of the vein was returned to the right side of the heart. The perfused tissue thus received blood aerated by the lungs and the pulsatile pressure was supplied by the heart itself. While this form of perfusion gave satisfactory blood flow the study of an isolated organ was complicated by the metabolism of the heart and lungs in the circuit.

A significant advance was made by Richards and Drinker (76), in 1915, by their development of an oxygenating device. It consisted of an inverted one liter bottle with the bottom sawed off. The blood inlet tube entered the bottle from the top and was bent into an horizontal circle provided with a number of holes in the side so that blood would spurt out through these holes outwards onto a cylinder of muslin or silk, arranged to suspend between the circular inlet tube and the wall of the bottle. Oxygen was supplied by means of a U-tube, one arm of which lay inside the muslin cylinder

and the other outside with the oxygen escaping through holes in the arms of the U-tube. Thus as blood flowed along the muslin in a thin film it came in contact with oxygen thereby becoming oxygenated. The oxygenated blood was removed through the mouth of the bottle. No foaming of blood was observed. Using a piston kind of pump and this oxygenating device they attempted the perfusion of the central nervous system of cats and the kidney of dogs but did not obtain a satisfactory blood flow. A similar oxygenator was introduced by Hooker (36), in 1915, wherein a rubber disc was rotated inside a bell jar by means of a hub and a pulley. Blood was allowed to fall on this disc and was thrown against the walls of the bell jar and ran down in a thin film thereby becoming oxygenated by the contained air or pure oxygen. Another device for oxygenation is letting the blood flow into a shallow trough and exposing films of blood to oxygen by means of a number of vertically rotating circular discs partly immersed in blood. The Hooker-Richards-Drinker oxygenator with modifications is by far the most predominently used.

Two kinds of pumps have been used in general, 1) the piston-type, 2) the suction type. The fluid in both is drawn into a chamber by means of a partial vacuum and is then forced out through an outlet, the flow being maintained in the desired direction by means of suitable valves.

A large number of apparatus have been described in the literature for the perfusion of a number of organs viz. rat pancreas by Long (50), kidney by Brodie (13), Nicholz and Verney (20), Hemingway (32), Janeway, Richardson and Park (39),

Richards and Plant (77) and Hooker (35); liver by Kapinov and Boricourt (44), testis by Peff et al (63). Other apparatus for the perfusion of various organs are described by Holgate (34), Allen (1), Costero (27), Steward (89), Green (29), Reynolds (75), Delli-Gaimini (26), Thomas (92), and Fleisch (21).

Perfusion Fluid

The physiological perfusion of any isolated organ with whole blood was found by almost all early workers to be a matter of extreme difficulty. The most important difficulty was the failure to obtain a satisfactory rate of blood flow through the organ. The presence of some vasoconstrictive substance was suspected and hence a departure was made from the use of blood rendered uncoagulable to an artificial medium. Isotonic sodium chloride, glucose-saline, Ringer-Locke and Tyrode solutions have all been tried with conflicting reports as to their success in maintaining normal physiological activity as a blood substitute. These artificial media which contain only crystalloids in solution are not able to exert the colloidal osmotic or "oncotic" pressure on which the maintenance of water balance largely depends. Hence, although a satisfactory flow of the perfusate was obtained through the organ, edema developed rather quickly. In order to overcome this undesirable effect other blood substitutes have been used e.g. 1) blood plasma and serum 2) hemolysed blood and 3) hemoglobin-Ringer solution. Blood plasma and serum although widely used in perfusion studies can be excluded as a suitable media for

the study of biochemical and physiological activities of organs and tissues as they are incapable of transporting and supplying the oxygen necessary for the normal functions of the tissues.

Hemolysed blood was used in a number of early perfusion studies. Opinion regarding the feasibility of the use of this medium for perfusion experiments are contradictory. Most of the workers found it to be toxic. Ringer (78) in his studies on salt balance used a solution of dried beef blood in water, diluted with five parts of saline solution, for the perfusion of frog hearts. He found no toxic effect on the frog heart due to the disintegrated cells. Similar non-toxicity of laked blood has been reported in the literature and has been reviewed by Emberson (2). The consensus of opinion was that the toxicity was associated with the stromata and not the hemoglobin. Schmidt (80) separated the stromata from hemolysed blood by centrifugation and showed that the stromata alone were responsible for the acceleration of coagulation of blood. That the toxic effect of hemolysed blood could be completely prevented by the removal of stromata was shown by a number of workers (2).

Emberson and Loper (3) investigated the various media for the perfusion of mammalian salivary glands. The use of serum or defibrinated blood, although preventing the development of edema, did not give a satisfactory perfusion, apparently due to the presence of a vasoconstrictor substance or substances. They then developed a perfusate consisting of 14% washed hemoglobin suspension in Ringer-Locke solution and obtained satisfactory rates of blood flow. Because of the absence of red cells and

the Donnan equilibrium they found that oxygen bound by hemoglobin was not easily made available to the tissues but concluded that this disadvantage was more than compensated for by other advantages. The hemoglobin in this preparation performs a dual role; 1) that of maintaining a colloidal osmotic pressure and 2) of transporting oxygen to the perfused organ. Smyth (87) used hemoglobin-saline in his studies and found that the solution could be used repeatedly, if kept sterilized. The hemoglobin-Ringer Locke solutions may be satisfactory for the perfusion of small organs but when dealing with large organs such as the bovine mammary gland the preparation of such large amounts of hemoglobin that would be required renders this medium cumbersome. Secondly, oxygen supply cannot be expected to be adequate, in the absence of cells, for an active gland, especially with a mechanical lung.

Blood as a Perfusion Fluid

Blood undoubtedly is the most desirable medium for perfusion, especially when the normal metabolism of an organ or tissue is under investigation. Being a heterogeneous medium, consisting of a large number of known and unknown chemical constituents, it suffers from many technical disadvantages. Besides the addition of a non-toxic and physiologically inert anticoagulant, care must be taken to minimize hemoconcentration, foaming, fibrin formation, hemolysis and trauma to the other cellular elements.

No clear cut observations were made on the vasoconstriction produced by whole blood until a continuous perfusion circuit was established with a mechanical pump and an oxygenator. The

probability of a chemical agent causing vasoconstriction was first mentioned by Hooker (36) who showed that the vasoconstrictive property of blood appeared to be associated with the process of clotting. A systematic study of the vasoconstrictor was made by Janeway, Richardson and Park (39) in 1918. They used a piece of carotid artery for the assay of vasoconstrictor substances. The technique consisted of passing the fluid, containing the substance to be assayed, through the carotid artery maintained at constant temperature in a bath and the recording of the constriction of the artery. Using this technique they were able to detect epinephrine in Ringer-Locke solution in a concentration of one part in 25,000,000 or even in 50,000,000. They found no constrictor action in citrated blood or plasma. Blood platelets of ox, dog and pig yielded an extract which had a powerful vasoconstrictor action which was not obtained from either erythrocytes or leucocytes. This vasoconstrictor substance was distinct from epinephrine; was not a protein and appeared to be a crystalloid. This substance, although present in coagulated blood, was not dependent on the actual formation of blood clot for its production, nor was it related to any of the factors concerned in the coagulation of blood, with the possible exception of thromboplastin.

Starling and Verney (88) used live heart-lung for the perfusion of kidneys and reported good rates of flow of defibrinated blood. Nicholz and Verney (20) investigated the failure of kidney perfusions with mechanical pumps by perfusing the kidney alternately with a mechanical pump and the heart-lung preparation. It was found that the pump gave progressively decreasing rates of blood flow, while the heart-lung or a

combination of heart-lung and the pump gave a four-fold rate of flow over that obtained with the pump alone. They concluded that heart-lung tissues must be removing the vasoconstrictor substance, "vasotonin", present in the defibrinated blood. An unlikely possibility was the addition of some vasodilator by the heart-lung preparations. According to these workers the inclusion of the lungs in a perfusion system was essential for two reasons, 1) they act as mechanical filter and 2) "the changes in the physicochemical state of the blood due to the absence of the lungs and the presence of the oxygenator may lead to the formation of particles injurious to the organ". Hemingway (32), in 1931, investigated the detoxification of vasoconstrictors by the lungs. He found that toxic properties developed in the blood on stagnation for two minutes but were rather quickly removed by lungs. The addition of defibrinated blood which had been standing for a while to blood already perfusing at a steady rate through the kidney and lungs of a dog causes vasoconstriction of both the kidney and pulmonary vessels but detoxification took place very quickly. In one of their experiments they used kidney alone to test whether the kidney tissue was able to remove the "vasotonin" partially or wholly. They obtained an initial rate of flow of 132 ml./min. which rose to 190 ml./min. in six minutes. At this point the lungs were added to the circuit when temporary slowing of the rate of flow was observed, no doubt due to the addition of stagnant blood present in the pulmonary vessels, followed quickly by a rise which became steady at between 177-184 ml./min. Removal of lungs from the circuit after a steady rate of flow was attained never caused

the development of vasotonins.

Extensive filtration of blood prior to and during the course of perfusion in the perfusion circuit was recommended by Anrep and Hausler (5) and Daly and Thorpe (19) after their investigation of the perfusion of mammalian hearts. Daly and Thorpe employed cambric filters of 50-100μmesh for filtration and emphasized the necessity of such thorough filtration, especially if the lungs are not included in the circuit.

Bayliss and Ogen (9), in 1933, using dog kidneys, conducted an investigation on the characteristics of the production of vasotonins in the blood by the measurements of perfusion pressure. No difference in relative toxicity was found between heparinized and defibrinated blood. The vasoconstrictive property developed within two minutes of the shedding of blood from the animal, with no significant increase in activity up to one and one-half hours to two hours regardless of temperature. Perfusing a kidney with blood within this time of the shedding of the blood caused the disappearance of "vasotonins" in about ten minutes. Blood kept longer developed a "permanent" vasoconstrictor which was never destroyed by the tissues on perfusion, but was destroyed automatically in 48-120 hours, whereas the "temporary" activity persisted. Defibrinated blood circulated through a pump-lung-kidney preparation lost both the "temporary" and "permanent", constrictive activities but on standing for one hour developed the "temporary" activity but not the "permanent" one. In their studies they did not

find the lung perfusion necessary for detoxification, as the kidney itself was able to destroy the vasoconstrictors; and provided all the blood passed through the kidney once, a satisfactory perfusion rate was obtained afterwards. In contradistinction to the observation of Daly and Thorpe (19), the inclusion from the beginning of the perfusion of cambric filters in the circuit did not result in any increased rate of blood flow.

Another vasoconstrictive or vaso-pressor system has been investigated by Page and coworkers (65). The kidney secretes a substance called renin and the production of the latter is particularly marked under hypertension (30). Renin is believed to be a proteolytic enzyme and varies in composition from species to species. For the study of vasoconstriction by this system Page (64) developed the technique of perfusion of rabbit's ear. The rate of flow of blood through the ear was recorded and any variation due to the injection of suspected vasoconstrictors noted. This procedure of assay has been used in extensive studies by Page and coworkers (65) and Landis et al (47). Purified renin was found to exhibit no vaso-pressor properties in Ringer-Locke solution but when the fraction of the plasma, "pseudoglobulin", was added interaction occurred and a substance with vasopressor properties was produced. A thermostable substance which was called angiotonin was isolated. Angiotonin itself was found to be non-vasoconstrictive as such but in the presence of an angiotonin-activator present in blood plasma a substance was

produced with marked vasoconstrictor activity.

The vasoconstrictive action of shed blood was investigated in great detail using the rabbit ear perfusion technique by Landis et al (47). Defibrinated blood was found to induce a marked vasoconstrictive effect. On comparing the rates of flow of blood drawn into heparin and drawn into heparin after previously heparinizing a donor rabbit, it was found that the preheparinized blood maintained a steady rate of flow over a period of nearly two hours, while the rate obtained with blood drawn into heparin declined gradually until it almost ceased in about forty minutes. In a second series of trials, the perfusion was begun with preheparinized blood and then shed blood or its various fractions were added to the perfusate after a steady rate of flow was obtained. Defibrinated blood, defibrinated blood containing heparin and blood serum were found to have a pronounced vasoconstrictive effect, the blood flow ceasing almost instantaneously. The addition of heparinized whole blood, heparinized plasma, preheparinized whole blood and preheparinized plasma did not result in any significat adverse effect, the preheparinized plasma showing no trace of activity. The study showed that heparin itself had no effect, harmful or otherwise. Slight cooling of the ear by the opening of the incubator door resulted in a lowering of the rate of blood flow, according to these workers, but it is disputed by Page (65). The work of Landis et al (op. cit.) appear to corroborate the suggestion of Janeway et al (39) that the development of vasoconstriction in shed

blood was somehow related to the disruption of blood platelets. A crystalline substance was isolated by Rapport et al (73) which had twice the activity of epinephrine, as assayed by rabbit ear perfusion. Its elementary analysis corresponds to the formula $C_{17}H_{30}N_6$ or $C_{14}H_{27}N_5$ and appears to be present in the form of a sulphate salt. Whether this is the substance that has been identified by the pharmacological action as "vasotonin" is not clear. It appears to be distinct from the vasopressor, the activated-angiotonin. It seems more than likely that there are a number of vasoconstrictor or potential vasoconstrictor substances. A bronchoconstrictor substance, "brochotonin" has been reported to be present in the blood of some species, e.g., dog and guinea-pig, but is not found in that of the monkey.

Although it is generally recognized that most tissues are capable of destroying the "vasotonin" and that a lung perfusion was not essential for a good rate of blood flow through other organs, Hebb and Linzell (31), in 1951, found that a nearly ten-fold increase in the rate of blood flow through the mammary glands of dogs, goats and cats was obtained with the inclusion of the lungs in series in the perfusion unit. With the increase in the rate of blood flow a corresponding decrease in the perfusion pressure was also noted.

Oxygenator

For the oxygenation of blood different workers have used different gas mixtures viz. air by Brodie (13) and Jacobj (38); pure oxygen by Bjork (11) and Petersen et al (68) and Peeters

and Masseart (66); and mixtures of 1% and 5% carbon dioxide in oxygen by Hebb and Linzell (31). The latter found that a 5% mixture of carbon dioxide in oxygen gave better results. Stokes and Gibbon (91) used 96% oxygen and 4% carbon dioxide for oxygenation prior to perfusion. They observed a rise in pH, up to as high as 8.5, when pure oxygen was used. On the other hand the use of their gas mixture, during the perfusion, produced an acidic condition, the pH going down to 6.6 from 7.4. On the basis of these results, they used the gas mixture for the oxygenation of blood prior to the perfusion and pure oxygen during the perfusion in order to keep the pH of the blood within the physiological range. Bjork (11) also found that the use of 100% oxygen caused an uncompensated carbon dioxide deficit accompanied by a rise in pH.

General Comments

From a survey of the literature it is obvious that a great deal of confusion persists regarding a physiological perfusion of an isolated organ. There are far too many perfusion apparatus described for the results obtained. On the other hand, a number of workers have presented data using this technique but have not paid attention to the conditions of the perfusion, which makes the evaluation of the data very difficult. As mentioned by Belt, Smith and Whipple (10), certain criteria of the normal physiological state of the organ being perfused need to be followed. They may be: 1) the rate of flow of blood as an indicator of tissue normalcy(any injury to the tissue is invariably

accompanied by a decreased rate of blood flow through that part) 2) the functional activity of the organ such as the measurement of urine production by the kidney; milk secretion by the mammary gland, 3) utilization of oxygen by the organ and 4) the production of edema, congestion or hemorrage.

The factors maintaining an organ in its physiological state are numerous and a perfusion of the organ is no better than its weakest point. If any one of the factors affects the organ adversely, perfection of all the other factors will not remedy the defect.

EXPERIMENTAL PROCEDURE AND RESULTS

The Perfusion Apparatus

The perfusion apparatus used in the early part of the studies was essentially similar to the one described by Petersen, Shaw and Visscher (69).

The blood was drawn by the pump, which constituted the mechanical heart, through a glass coil kept immersed in a thermostat maintained at 39° -40° C. The "heart" consisted of a glass tube (sleeve), approximately 1½" in diameter and 6" in length, tapered at both ends to 1" in diameter, and provided with a side tube. A thick rubber tube, 1" in diameter and 6" in length, was passed through the glass sleeve and was kept in place by means of tightly fitting rubber stoppers pushed into the ends of the rubber tube, so that the outer wall of the rubber tube was pushed tightly against the inner wall of the ends of the glass sleeve. A short glass tubing was passed through each of the stoppers, one of them carrying an inlet valve inside the rubber chamber and the other an outlet vlave outside the chamber, so that the blood would be allowed to enter the chamber through one end and leave the chamber only through the other end. The valves consisted of eye-dropper bulbs which were slit longitudinally. The side tube of the glass sleeve was connected through a milking machine interrupter (pulsator) to a vacuum pump. A vacuum in the line caused the expansion of the rubber chamber drawing the blood into the chamber from below; on the release of the vacuum the rubber chamber returns to its

normal size compressing the blood inside the chamber, which closed the inlet valve and forced the blood through the outlet valve into the arterial tubing. The latter was connected by means of a suitable canula to the mammary artery of the udder kept in a net suspended from the stand.

The blood flowing out of the veins of the gland was collected through a filter cloth in a funnel and passed into the oxygenating chamber by gravity.

The oxygenator consisted of an inverted 10 gallon glass carboy with a hole of approximately 1½" in diameter drilled in the center of the bottom. The oxygenator was provided with an arrangement for the distribution of the blood along the inner wall of the chamber and is shown in figure I. A glass tube 3/4" in diameter and approximately 3" in length was fitted into the rubber stopper, closing the hole in the bottom of the carboy, so that one end of the glass tube was flush with the lower side of the stopper. To the other end was fixed another stopper, approximately 5" in diameter which closed the end of another tube, approximately 6" in length and provided with the blood inlet tube which was fused through its wall. The blood distributor consisted of a glass thistle funnel to the stem of which was fused a glass tubing through the middle of the cup, so that glass tubes extended above and below the cup of the funnel. The tube extending below passed through the glass jacket connecting the oxygenating chamber and the distributor head. The upper end of the tube was attached to a motor. The cup of the funnel rested on a ball bearing which was fixed in a shallow

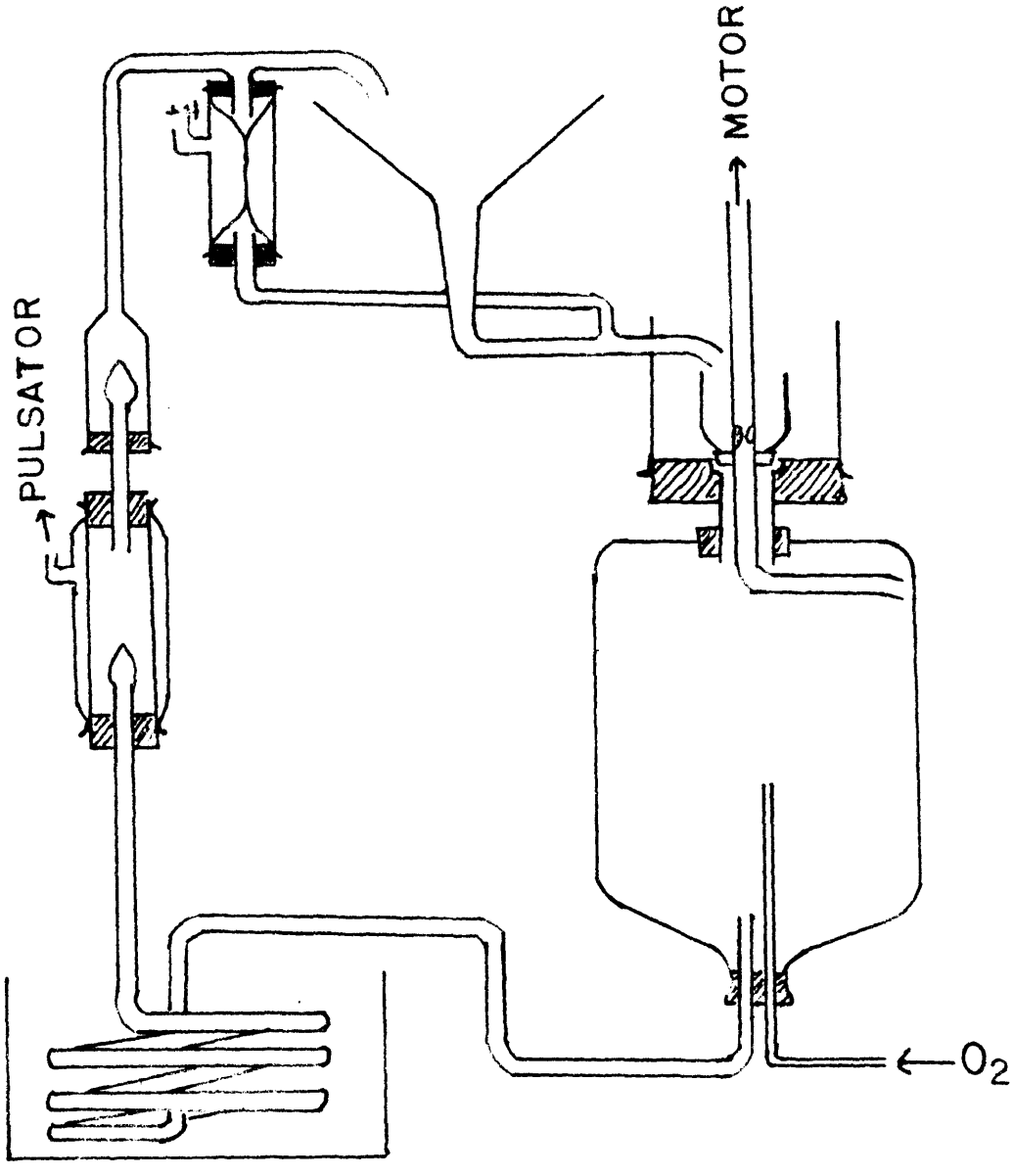


Figure I. A diagrammatic sketch of the perfusion apparatus.

depression made in the rubber stopper and which supported the weight of the entire blood distributor system. Inside the chamber the inlet tube from the funnel was connected to another glass tube bent at a right angle, the end of which extended nearly up to the inner wall of the carboy. The venous blood poured into the funnel and entered the stem of the funnel through a number of holes in the central tube. As the funnel was rotated by the motor the venous blood pouring into it was distributed evenly along the wall of the carboy and became oxygenated by the oxygen inside the chamber. Oxygen or any gas mixture used for oxygenating the blood was bubbled through water to saturate it and entered the chamber through the mouth of the carboy. Saturation of the gas with moisture was necessary in order to prevent the dehydration of the blood. The oxygenating chamber also formed the reservoir whence the blood was drawn by the pump.

The pressure regulating device consisted of an arrangement similar to the "heart", except for a gooch rubber tubing substituting the thick rubber tube, and was kept collapsed by maintaining a known pressure (100-110 mm. of mercury) on it. It was connected to the arterial tube by means of a T-tube. Blood flowing through the artery at a pressure in excess of the 100-110 mm. mercury caused the distension of the gooch tubing and the excess blood was diverted from the arterial system into the "lung" chamber. Valves were found to be unnecessary in this system.

Care and Treatment of the Blood

Six to eight liters of blood were collected in a wide mouth glass bottle or an enamel bucket with a thick paraffin coating to avoid the contact of the blood with the metal surface. Heparin or chlorazol fast pink B was used as the anticoagulant. After thorough mixing the blood was filtered through several filters consisting of cheese cloth and cheese cloth wrapped around cotton and Rapid Flo filters (Johnson and Johnson). The filtered blood was oxygenated prior to perfusion. After washing the udder with saline one of the arteries was cannulated and half of the udder was perfused. The data obtained with five perfusion experiments are presented in table I.

The data in table I show that the rate of blood flow obtained in the first four perfusions was considerably below the physiological rate. The onset of the vasoconstriction was quite rapid, the maximum rate of flow obtained at the initial stage decreased progressively. The last perfusion (No. 12) showed an initial increase in the rate of flow over a period of twenty minutes which subsequently declined during the next 25 minutes from a relatively high value of 625 ml/min. to 85 ml/min. Perfusion of the other half of the gland did not reproduce the above initial rate of flow and resulted in a marked decrease in the rate of flow; viz. 400 ml/min. was reduced to 120 ml/min. in five minutes. The relatively high rate of flow obtained (600 ml/min.) appeared to be due to the extremely short time lapse

between the shedding of the blood and the beginning of the perfusion. The poor results obtained by the perfusion of the other half of the udder shows that vasoconstriction in the blood had already developed.

Effect of Lung Perfusion

In order to test whether prior perfusion through the lungs have a favorable effect on the rate of flow of blood through the mammary gland, four perfusions were carried out after the blood had passed through the lungs. The data are presented in table 2. After filtration the blood was pumped through the lungs once or twice and then one half of the gland was perfused. A slight beneficial effect appeared to be indicated from the rates of flow which were significantly higher than those obtained in earlier perfusions without the use of lungs (table 1). Although the high rates of flow were not maintained for any length of time, the decrease in flow, in at least one perfusion, (perfusion 10) was not as rapid as obtained in the data reported in table 1. This could have been fortuitous so an additional study was made. Perfusion of one-half of the gland was initiated prior to the lung perfusion and after noting a decrease in the rate of blood flow, the flow of blood to the gland was discontinued and the blood was pumped through lungs twice. The blood was then perfused through the half of the gland not perfused before. A more nearly constant rate of flow was obtained over a period of 30 minutes. It was felt that the first half of the udder might have had the blood vessels blocked off

Table I

The Rates of Blood Flow in Perfusions without Prior Lung Perfusion.

Perfusion No.	Half gland perfused	Time-lapse between killing and perfusion (min)	Perfusion time (min)	Rate of blood flow (ml/min)
2	Right	90	0	-
			20	375
			30	0
	Left	185	0	-
			5	200
			20	220
			35	180
			65	190
3	Right	95	0	-
			10	250
			40	150
			70	72
4	Right	60	0	-
			10	184
			20	80
	Left	90	0	350
			6	230
			11	136
6	Right	140	0	-
			15	280
			25	200
			50	164
			65	72
12	Right	15	0	340
			10	510
			20	620
			30	200
			45	85
	Left	65	0	400
			5	120

Table 2

The Rates of Blood Flow in Perfusions with Prior Lung Perfusion

Perfusion No.	Time lapse between killing and perfusion (min.)	No. of passages through lung	Perfusion time (min)	Rate of blood flow ml/min
8	106	1	0	440
			3	500
			15	150
			30	100
			34	66
			70	35
			80	24
9	235	1	0	660
			8	280
			15	330
			30	270
			42	160
			50	100
			60	90
			75	48
10	40	2	0	-
			5	374
			9	480
			13	760
			20	780
			25	648
			35	480
			39	520
			50	400
			58	288
			67	180
			86	190
			95	136
			102	164
			110	140
			117	104
			135	70
11	105		0	-
			2	340
			7	320
			15	95
			20	100
			30	75
			34	35
			40	24

and that the second half of the gland might have given the same results with blood not perfused through lungs. So the first half was perfused again. The data are shown in table 3. It will be seen that the gland now gave a rate of flow greater than obtained prior to lung perfusion, and was maintained for 30 minutes.

Another perfusion was carried out in which the lung was perfused prior to the mammary gland perfusion. After the rate of flow had slowed down considerably the effect of the lungs on the detoxification of blood was tested. The data are shown in table 4. No favorable effect was observed.

That the lung perfusion does not always result in a better rate of flow was shown in perfusion No. 16. One half of the udder was perfused before lung perfusion and an initial rate of flow of 240 ml/min. of blood was obtained which declined in 10 minutes to 30 ml/min. The lung was then perfused so that the seven liters of blood used passed through the lung twice. When the other half of the gland was perfused the rate of flow was not increased.

Effect of Mode of Suspension of the Gland

In the last mentioned perfusion (No. 16) after the blood flow had almost ceased the gland was suspended upside down by means of the teats to determine whether the weight of the udder caused the collapse of the blood vessels when it was suspended in a bag or a net. In the previous perfusions the mammary gland had always been suspended in this manner. A flow rate of 500 ml/min. was obtained. An attempt was then

Table 3

The Effect of Lung Perfusion on the rate of Blood Flow through the Mammary Gland.

Perfusion No.	Time-lapse between killing and perfusion (min)	Perfusion time (min)	Rate of Blood Flow (ml/min)	Remarks
5	80	0	170	
		20	110	
		32	130	
				Blood pumped through the lung 2 times and a new udder perfused
		0	-	
		5	280	
		18	250	
		24	250	
				The first gland perfused again
		0	-	
		5	200	
		15	200	
		25	200	

Table 4

The Effect of Lung Perfusion after the Development of Vasoconstriction.

Perfusion No.	Time-lapse between killing and perfusion (min)	Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
7	150	0	-	The blood was pumped through the lung twice prior to gland perfusion
		5	600	
		20	780	
		45	710	
		55	650	
		58	250	
		65	370	
		72	260	
		80	100	
		85	110	
		90	90	
				The lung was perfused again to give two complete passages of the blood and then the same gland perfused.
		0	70	
		15	70	

made to suspend the gland in such a way as to simulate the natural condition. This was done by suspending the udder from the median suspensory ligament by means of three or four hooks. Two additional hooks were used on either side of the gland to stretch the skin around the teats so as to distribute the weight of the gland more uniformly. The data on two perfusions are shown in table 5. The time-lapse between the slaughter and the beginning of the perfusion was nearly two hours in each of the perfusions, 17 and 18, (table 5). The blood was not passed through a lung. Perfusions 16, 17 and 18 show the importance of the manner of suspending the mammary gland, and that conditions approaching the natural state in the body favor a better perfusion rate. The data also show that a prior lung perfusion is not essential for a good rate of flow.

Nature of Arterial Flow

In a number of preliminary studies it was observed that blood passed through the gland under hydrostatic pressure at a more uniform rate over a fairly long period of time. It was felt that the pump used in these studies might have a detrimental effect on the perfusion as the flow obtained from it was not physiological inasmuch as the pump delivered blood in an interrupted manner. It thus did not duplicate the flow obtained in the body by the pumping action of the heart, which supplies a steady flow of blood with pulsation superimposed. The pump supplied blood in spurts followed momentarily by complete cessation of the flow. In order to

Table 5

Rates of Blood Flow on Suspending the Udder by the Median Suspensory Ligament.

Perfusion No.	Perfusion time (min)	Rate of blood flow (ml/min)
17	0	320
	20	320
	30	340
	60	360
	70	400
	85	472
	100	480
	115	560
	130	536
	145	480
	150	400
	160	400
18	0	700
	30	640
	45	640
	75	680
	105	720
	120	780

maintain a constant blood pressure the blood was kept in a reservoir at a height of 5 to 6 feet and perfusions were carried out using this head of blood. All the other conditions remained the same e.g. temperature of the blood, suspension of the udder, amount of anticoagulant, filtration etc.

The data obtained under these conditions in a perfusion (No. 32) are shown in table 6. The time-lapse between the shedding of blood and the beginning of perfusion was approximately one hour. It also shows that once a good rate was obtained and maintained for some time changing to the pump had no deleterious effect, perhaps because the vascular system of the gland is more sensitive to variations in blood pressure at the beginning of the perfusion. This may be due to the gland having gone without blood supply in the interval between the shedding of the blood and the beginning of the perfusion. A further trial (perfusion 33) was conducted in which the normalcy of the blood was established by perfusing a mammary gland by means of gravity flow. A rate of 400 ml/min. was obtained which was maintained for a considerable time. Using the same sample of blood the perfusion of another gland was attempted using the pump, all the other conditions remaining the same. An initial rate of 400 ml/min. declined to almost nothing within five minutes. This experiment indicated that the unnatural nature of blood flow obtained by using this pump had a definite harmful effect on the rate of perfusion.

Table 6

Rates of Blood Flow under 5 feet of Hemostatic Pressure.

Perfusion No.	Perfusion Time	Rate of Blood Flow (ml/min)	Remarks
32	0	-	
	10	600	
	20	666	
	30	750	
	40	750	
			Gravity flow changed to the pump
	50	750	
	60	750	

Effect of Hyperventilation

While investigating the rates of blood flow obtained with various samples of blood through the mammary gland it was noticed that a venous sample of blood passed through the mammary gland at a rate faster than a well oxygenated sample. It seemed desirable to follow this accidental observation further and to see whether exhaustive oxygenation using pure oxygen actually had an adverse effect on the rate of perfusion.

As much of the blood as could be obtained from one cow was taken and divided into two parts. One part was oxygenated thoroughly with pure oxygen, the time of oxygenation varying from 45 to 60 minutes in the perfusion trials. Perfusion of the mammary gland was started with the unoxygenated sample of blood and after a steady rate was obtained the perfusion

fluid was changed to the oxygenated sample of blood. The rates of flow were measured at frequent intervals. As the oxygenated sample of blood was going through the gland the venous or poorly oxygenated sample was oxygenated. The oxygenated sample that had gone through the gland once was used as representing the poorly oxygenated sample. Thus the gland was perfused alternately with well oxygenated and poorly oxygenated ("venous") blood samples. Gravity flow was used throughout these studies. The time-lapse between the shedding of the blood and the beginning of the perfusion varied from 40 to 60 minutes. Data on three perfusions (No. 45, 48, and 52) are given in tables 7, 8 and 9.

These data show that a hyperventilated sample of blood had a definite harmful effect on the perfusion rate. Blood samples were analyzed manometrically (68) for their oxygen and carbon dioxide contents in perfusion 52. These data are presented in table 10.

It can be seen that the hyperventilated sample is very nearly saturated with oxygen. The carbon dioxide contents were correspondingly low.

Effect of the use of 5% CO_2 and 95% O_2

The low values for carbon dioxide content of the hyperventilated blood causing vasoconstriction in the vascular system of the mammary gland suggest the possibility that the depletion of carbon dioxide caused by the use of pure oxygen, for the oxygenation of blood, might be responsible for the vasoconstrictive effect. Two perfusions

Table 7

The Effect of Hyperventilation on the Rate of Blood Flow (Perfusion 45)

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	390	---------- venous blood
5	250	
8	170	
10	210	
14	160	
15	200	
		---------- Oxygenated blood
18	140	
21	120	
24	120	
28	100	
31	90	
35	75	
40	70	
45	70	
50	103	----------venous blood
54	190	
58	210	
63	340	
70	350	
73	380	
77	360	
		---------- oxygenated blood
80	330	
83	260	
87	210	
89	170	
91	135	
93	135	
95	120	
97	105	
		----------venous blood
99	110	
101	110	
103	100	
105	120	
107	200	
109	300	
111	310	
113	360	
128	550	
131	580	

Table 8

Effect of Hyperventilation on the Rate of Blood Flow (Perfusion 48)

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	180	----------venous blood
3	180	
4	210	
6	240	
9	250	
15	275	
18	320	
20	335	
		----------oxygenated blood
22	390	
24	280	
25	125	
26	100	
28	80	
30	80	
32	90	
35	90	
37	90	
		----------venous blood
40	85	
41	85	
43	150	
45	250	
46	300	
48	320	
the perfusion was stopped and resumed after 40 minutes with the same set up.		
49	400	
51	360	
53	350	
56	400	
58	340	
		----------oxygenated blood
64	100	
66	250	
68	350	
70	360	

Table 9

Effect of Hyperventilation on the Rate of Blood Flow (Perfusion 52)

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	-	---------- venous blood
2	425	
5	460	
8	560	
12	575	
14	610	
20	610	
29	610	
		---------- oxygenated blood
30	400	
31	250	
33	240	
35	220	
37	210	
42	190	
		---------- venous blood
43	220	
44	450	
46	600	
50	600	
		---------- oxygenated blood
52	300	
		---------- venous blood
61	480	
63	525	
69	600	
81	600	
85	620	
		---------- oxygenated blood
87	360	
89	310	
90	300	
		---------- venous blood
92	450	
95	610	
104	600	
113	600	
116	610	
121	640	
126	625	

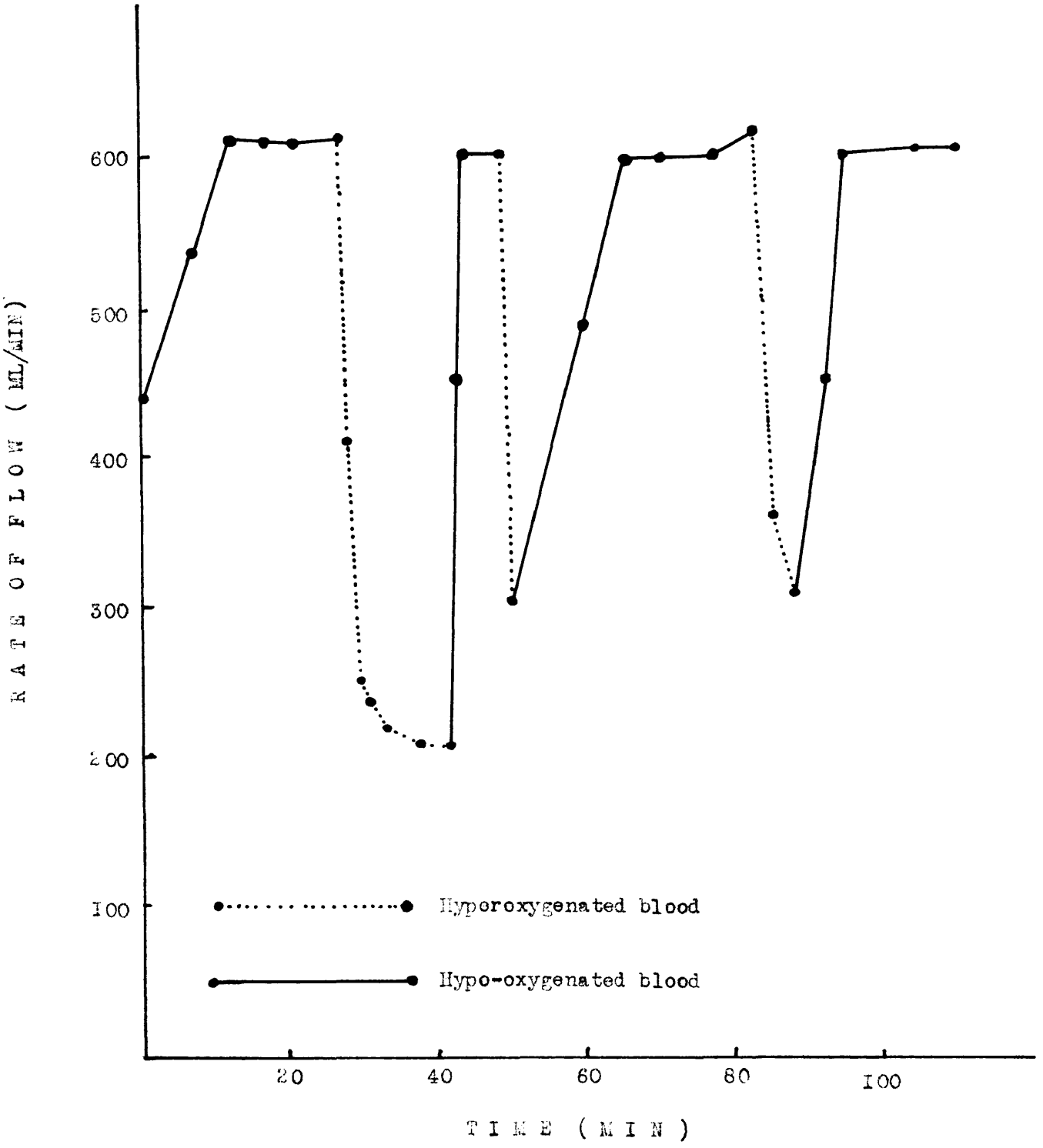


Figure 2. The effect of hyperventilation on the rate of blood flow.

Table 10

Oxygen and Carbon Dioxide contents of blood samples in Perfusion 52.

Sample No.	Nature of blood	Perfusion time when sample drawn (min)	O_2 vol.%	CO_2 vol.%
1	blood as received from the animal	-	6.04	49.76
2	blood as received from the animal	-	5.68	44.33
3	hyperventilated	25	18.27	23.08
4	hyperventilated, blood passed thru the gland once	75	9.77	30.46
5	hyperventilated	90	18.48	18.37

of the bovine udder were carried out to see whether the same vasoconstrictive effect could be produced when a gas mixture containing 5% carbon dioxide and 95% oxygen was used for the aeration of blood. The procedure adopted was similar to those described previously for the study of the effect of hyperventilation except that the gas mixture was used instead of pure oxygen. The data are presented in table 11 and 12.

The data presented in the above two tables indicate that the vasoconstriction obtained by using pure oxygen for oxygenating blood was abolished by using 5% carbon dioxide in oxygen, but this was not beyond dispute as the data obtained are marked with fluctuations. Hence a third perfusion was performed in an attempt to establish whether the use of the gas mixture would obliterate the vasoconstriction produced by the use of pure oxygen. In this perfusion (No. 38) the blood obtained from the cow was divided into three batches, one oxygenated with pure oxygen, a second with 5% carbon dioxide in oxygen and a third was used as venous blood. The rates of the flow obtained with these three samples of blood are shown in table 13.

The mammary gland perfusion was commenced with venous blood and then with blood oxygenated with pure oxygen to bring about vasoconstriction. This was followed by perfusion with blood oxygenated with 5% carbon dioxide and unoxygenated blood to see whether both of these bood samples relieved the vasoconstriction to the same extent. It is seen from the data that although the initial high rates of flow were not obtained, both the venous blood and the blood

Table 11

The Rates of Blood Flow Obtained with Blood Oxygenated with 5% Carbon Dioxide and 95% Oxygen Mixture and Unoxygenated Blood (Perfusion 57).

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	-	---------- venous blood
1	150	
5	200	
10	225	
15	250	
19	275	
24	300	
		---------- oxygenated blood
40	250	
50	275	
		---------- perfusion discontinued for lack of oxygenated blood for 15 min. and commenced again with oxygenated blood.
70	360	

Table 12

Rates of Blood Flow Obtained with Blood Oxygenated with 5% CO_2 and 95% O_2 and unoxygenated Blood (Perfusion 66).

Perfusion time (min)	Rate of flow (ml/min)	Remarks
0	-	---------- oxygenated blood
5	350	
10	250	
		---------- venous blood
20	375	
35	350	
		---------- oxygenated blood
50	325	
90	225	
		---------- venous blood
105	200	
110	150	
115	150	

Table 13

The Rates of Blood Flow Obtained with Blood Oxygenated with pure Oxygen, 5% Carbon Dioxide and 95% Oxygen Mixture and Unoxygenated Blood (Perfusion 68)

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	-	---------- 5% CO_2 blood
5	500	
13	575	---------- venous blood
16	550	
20	550	
29	470	
42	510	
		---------- O_2 blood
44	380	
47	260	
54	240	
56	250	
60	165	
		---------- 5% CO_2 blood
65	240	
69	240	
72	260	
		--------- venous blood
75	240	
78	290	
80	290	
85	290	
		---------- O_2 blood
90	145	
100	125	
104	125	
		---------- venous blood
110	260	
112	280	
114	290	

oxygenated with 5% carbon dioxide relieved the vaso-constriction produced by the sample oxygenated with pure oxygen to nearly the same extent. The data on the three perfusions showing the effect of 5% carbon dioxide in oxygen is shown graphically in fig. 3.

The hyper- and hypo-oxygenated blood samples in perfusion 66 were analyzed for their oxygen and carbon dioxide contents. The data are presented in table 14.

Table 14

Carbon dioxide and Oxygen Contents of Blood Samples (perfusion 66).

Sample	O_2 vol. %	CO_2 vol. %
Hyper-oxygenated blood	16.55	25.74
Venous blood	11.11	22.90

The degree of oxygenation of the blood does not seem to be markedly different from that obtained using pure oxygen.

Effect of Chloral Hydrate

Since the action of the blood hyperoxygenated with pure oxygen produces a vasoconstriction almost instantaneously, it appeared that the nerve fibres of the vascular system of the isolated mammary gland must still be active and that the

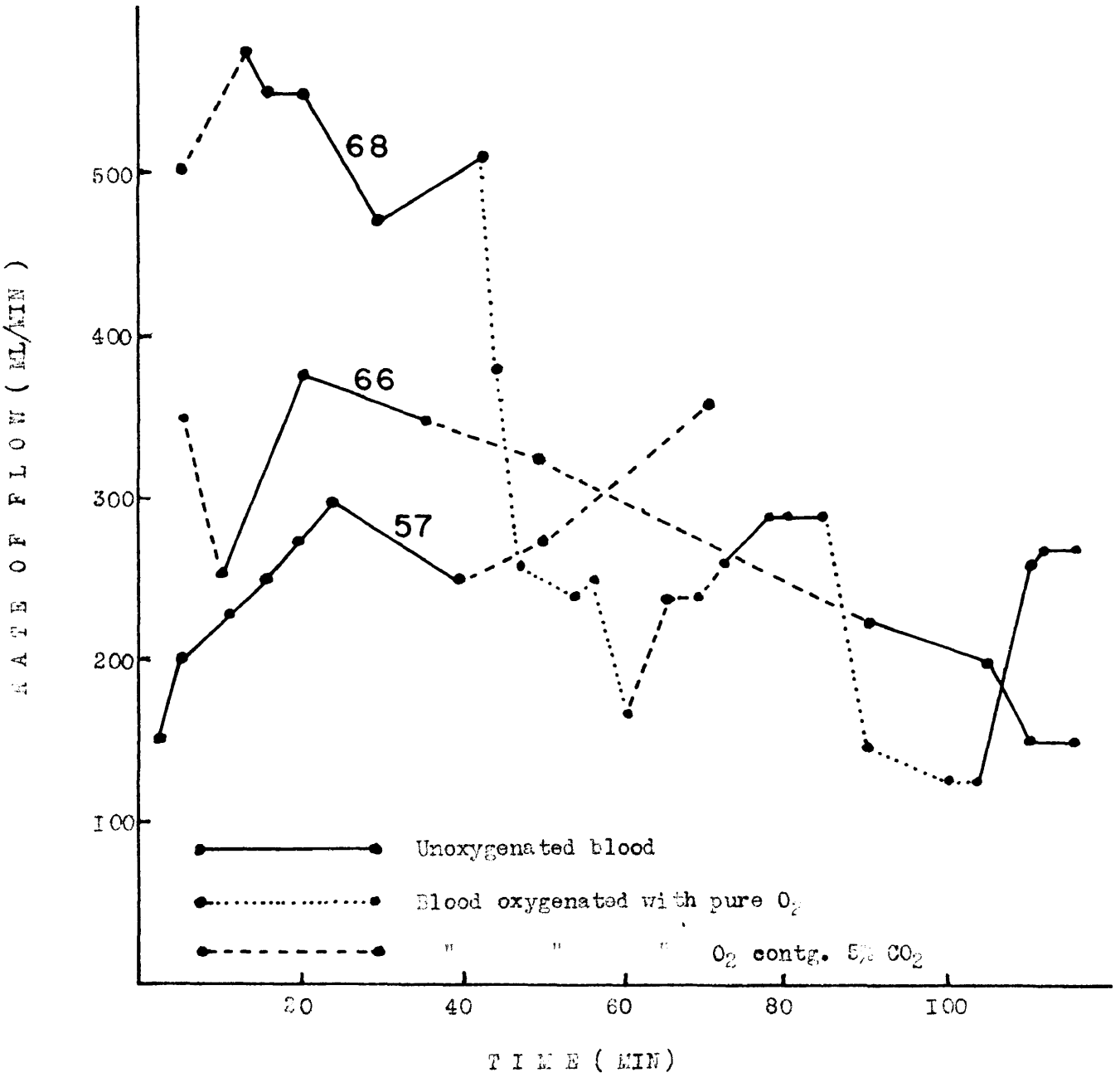


Figure 3. Rates of flow of blood oxygenated exhaustively with pure O_2, O_2 containing 5% CO_2 and unoxygenated blood. (Perfusions 57, 66 and 68)

entire vascular system of the gland may be effected through the medium of the nerves. perfusion was performed to determined whether the presence of chloral hydrate in the blood would prevent the development of the vasoconstriction by the oxygen-hyperventilated blood. The chloral hydrate is believed to narcotize or anaesthetize the nerves without effecting the metabolism of the organ. Ten grams of chloral hydrate dissolved in a minimum quantity of physiological saline was added to 12 liters of blood mixed thoroughly. The blood was then divided into two parts, one part being oxygenated thoroughly with pure oxygen. The rest of the procedure was the same as in the study of the effect of hyperventilation (perfusions 45, 47 and 52). The data are shown in table 15. As the data showed fluctuations difficult to interpret another perfusion study was made. The perfusion was begun with venous blood and after a steady rate of blood flow was obtained the vasoconstriction was established by perfusing the gland with blood hyperventilated with pure oxygen. The same hyperventilated blood containing 100 mgm. per cent chloral hydrate was then passed through the gland. The data are given in table 16 and figure 4.

Changes in the pH of Blood

The pH values of blood were determined in some of the perfusions from time to time. The initial pH values at zero time represents the values obtained on blood after oxygenation and proceeded for at least thirty minutes with pure oxygen. The data are shown in table 17.

Table 15

The Effect of Addition of Chloral hydrate to the Blood on the Rates of Blood Flow (Perfusion 54).

Perfusion time (min)	Rate of blood flow (ml/min)	Remarks
0	-	---------- venous blood
2	200	
5	450	
7	450	
10	500	
13	500	
17	550	
21	530	
		---------- oxygenated blood
26	500	
28	475	
30	450	
32	450	
		---------- venous blood
36	500	
38	480	
41	490	
45		

Table 16

The Effect of Addition of Chloral Hydrate to the Hyperoxygenated Blood on the Rates of Blood Flow (Perfusion 55).

Perfusion time (min)	Rate of flow (ml/min)	Remarks
0	-	---------- venous blood
3	300	
6	240	
10	250	
13	310	
17	350	
19	390	
22	400	
27	410	
		---------- hyper-oxygenated blood
30	275	
35	225	
37	200	
		---------- hyper-oxygenated blood containing chloral hydrate
43	375	
47	425	
51	425	

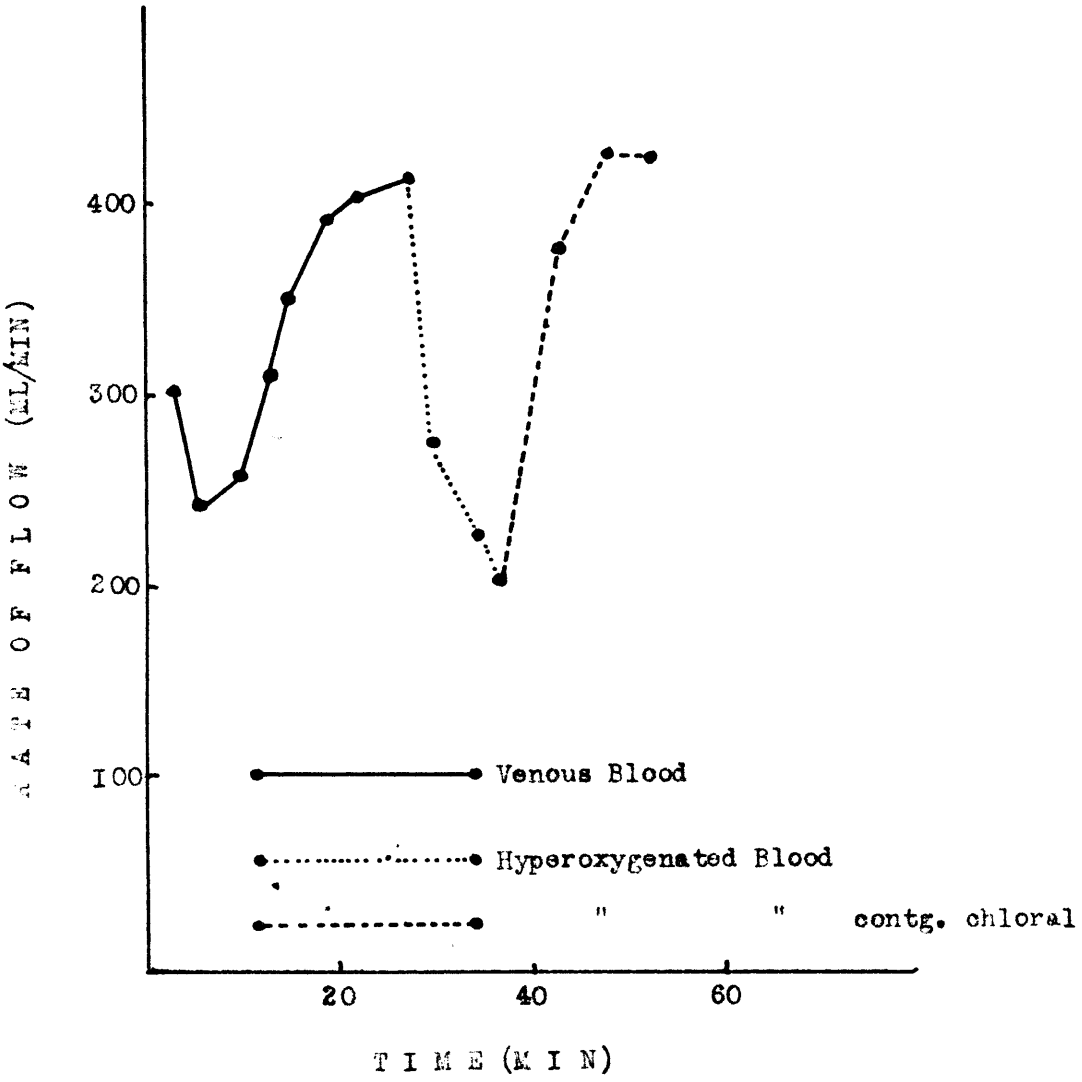


Figure 4. The effect of Chloral Hydrate on the vasoconstriction produced by hyperoxygenated blood.

Table 17

The Effect of Perfusion on pH of Blood.

Perfusion No.	Time (min)	pH of blood Arterial	Venous	A-V difference
3	0	7.10	-	-
	30	7.20	7.00	0.20
	60	7.05	7.05	0.00
	90	7.15	7.05	0.10
8	0	7.70	-	-
	35	7.50	7.15	0.35
	60	7.40	7.05	0.35
10	0	7.60	-	-
	20	7.63	7.55	0.08
	44	7.70	7.60	0.10
	85	7.70	7.53	0.17

DISCUSSION

The maintenance of an isolated organ in its physiologically functional state is the goal of the perfusion techniques. This task is beset with innumerable difficulties, mainly because of the necessity of maintaining blood in a normal physiological state if blood is used as the perfusate. Other media which are artificially prepared, even if composed of blood constituents, can at best be considered only a substitute for blood. Of the various blood substitutes used hemoglobin-Ringer-Locke solution is the closest, physiologically, to blood. This solution is capable of carrying out adequate respiratory function in live animals (4) and has the advantage of giving a good rate of flow in the perfusion of isolated organs. It suffers, however, from a great disadvantage when applied to the study of milk secretion by the mammary gland. All the constituents of blood that take part in milk secretion are not known and hence the preparation of an artificial medium complete in all nutrients, as far as requirements for milk secretion is concerned, is an impossibility. The use of artificial media lacking in one or more essential metabolite would be introducing complications and would defeat the purpose of perfusion, which at this stage is to study the normal activity of the isolated mammary gland and to determine the nature and quantities of catabolites removed from the blood for the syntheses of individual constituents of milk. Should a

complete knowledge of the various metabolites involved in the secretion of milk be available, artificial media might be used to study the effect of the level of individual metabolites on the nature of the secretion. But until such information is available the use of artificial media would be expected to give data which would be difficult to interpret since the knowledge of the metabolic reactions of the mammary gland is in the rudimentary stages. For instance, failure to get any milk secreted by a lactating mammary gland after maintaining a good rate of flow through the gland for an adequate period of time could be interpreted to mean that the abnormality was inherent in the gland itself and the failure of milk secretion was not due to the unavailability of all nutrient substances. An interpretation of even such a simple nature would be difficult when a further complication (the use of artificial media) is introduced to a little understood and already complex situation.

An adequate rate of blood flow through an isolated organ even if lower than the physiological rate is governed by a large number of factors, not many of which are clearly understood. Some of the factors affecting blood flow through the isolated organs are: 1) the development of vasoconstrictor substances in the blood, 2) the production of solid particles, probably due to coagulation, 3) the nature of the arterial flow and 5) factors associated with the extent of oxygenation and the oxygenating medium.

Factors (1) and (2) can be considered together. It can be seen from the data in tables 1 and 2 that the highest

sustained rates of blood flow were obtained in perfusions where the time lapse between the bleeding of the animal and the beginning of the perfusion was short (perfusion 10 in table 2 and 12 in table 1). The general pattern of the early perfusions was always the same, an initially high rate of flow showing down rather quickly until the blood flow almost ceased. Prior perfusion of blood through lungs did not indicated any significant beneficial effect in these studies. A slight beneficial effect was observed in perfusion 5 (table 3) but no effect in perfusions, 7 (table 4) and 16 (text, page 27). The effect of the lung perfusion has been ascribed to the filtering action of the lung and secondly, the detoxifying action on the vasoconstrictor substances. The ability of all tissues in detoxifying temporary vasoconstrictors is well known (9), and as long as the blood contains only temporary vasotonins they are destroyed by the tissues, but a permanent vasoconstriction produced in the blood due either to stagnation or agitation is not destroyed. These two distinct constrictive phenomena might explain the failure of the early perfusions. Because of the failure to obtain a good rate of flow initially, the blood was agitated in the pump to a greater extent thereby leading to the production of permanent vasoconstriction, which would depress the rate of flow further. Some of the causes of the initial low rate of flow might be the unnatural manner of suspension, the effect of the unnatural flow obtained by the pump by the blocking of the blood vessels of the organ by filterable substances or the excess secretion of epinephrine

due to excitation preceding killing.

The manner of suspension has a decided effect on the perfusion rate. This can be seen from the information gathered in perfusion 16. Perfusions 17, 18 (table 5) and 32 (table 6) show that once a good rate of blood flow was obtained through the gland initially, it continued for a long period of time even with the pump used in these studies. The latter has been found to have a definite detrimental effect (perfusion 33, text page 32). A good rate of flow through the system does not cause any violent agitation of the blood inside the "heart" in the apparatus used or any stagnation in any other part of the apparatus and consequently the entire quantity of blood would be maintained in a uniform state of motion. This might be expected to keep the blood free from vasoconstrictors, as all the blood is constantly passing through the gland, thereby detoxifying any vasoconstrictor(s) produced.

The harmful effect of the pump, as mentioned before, may be due to the non-physiological nature of the blood delivery causing an irritation of the blood vessels. It has been noticed frequently that rough handling of the artery causes it to contract and hence an intermittent blood flow being forced into the artery in sudden violent spurts, with even a negative pressure interspersed in between spurts, could be expected to cause considerable irritation and, consequently, constriction of the arteries and the other blood vessels. The blood vessels apparently are more sensitive at the

beginning of the perfusion than later, perhaps because of the occlusion of blood flow in the interval between the bleeding of the animal and the severance of the udder from the body. Most workers emphasize the utmost care in the removal of the organ to be perfused. The conditions under which the mammary glands of cows were obtained from the slaughter house can hardly be considered adequate for physiological studies of a precise nature. The stunning of the animal undoubtedly results in the liberation of considerable epinephrine, and hence the blood vessels of the animals at the time of bleeding may already be in a constricted state. The presence of epinephrine in the blood may also account for the lowered flow rates at the beginning of the perfusion. An idea of the extreme sensitivity of the mammary gland of goats can be obtained from the value, 7.45×10^{-14} g. of adrenaline per gram of mammary tissue found by Webb and Linzell (31) to produce vasoconstriction. The values for cats and dogs were of the same order.

The effect of hyper-oxygenation was an entirely unexpected factor affecting the rate of blood flow. From the data presented in table 9 and fig. 2, it can be seen that the response of the mammary gland to hyperoxygenated blood is almost instantaneous as evidenced by the rapid decline of the blood flow. The recovery of the blood following the use of hypo-oxygenated blood is equally dramatic. The suggestion from this observation that the nervous system was involved in some manner found confirmation in the results obtained with blood to which chloral hydrate had been

added (tables 15 and 16 and fig. 4).

In this connection the retention of the excitability of the vasoconstrictive nerve fibres, carried by the external spermatic nerves of the mammary gland, found by Heub and Linzell (31) throughout the duration of their perfusion is of interest.

The mode of action of this vasoconstrictive effect is perplexing. The oxygen contents of blood oxygenated with pure oxygen and oxygen containing 5% carbon dioxide (tables 10 and 14) are not significantly different, the difference being only approximately 2 ml. of oxygen per 100 ml. of blood. It is quite possible that at near the saturation point this slight difference may have marked influence.

The desirability of using a carbon dioxide-oxygen mixture has been mentioned by various workers (31, 91). Stokes and Gibson (91) found that the pH of the blood oxygenated with pure oxygen rose to as high a value as 8.5 whereas using 4% carbon dioxide in oxygen maintained the pH at about the same level. In their work oxygenation for approximately 30 minutes with pure oxygen increased the pH value in their experiment from 7.5 to 8.3 rising in the next thirty minutes to 8.5. No such wide fluctuations have been observed in the pH values determined in the course of this series of studies, where pure oxygen was used for oxygenation (table 17). The pH values obtained for arterial blood ranged between 7.05 to 7.7 with a mean decrease of 0.19 units for venous blood. It would appear from this that it is not the pH of the blood that regulates the rate of blood flow, since the blood which has

once traversed the gland usually goes through the gland at a markedly increased rate even though the pH has not changed significantly.

Part II

Acetate and Beta-hydroxybutyrate Metabolism of the Perfused Lactating Bovine Mammary Glands

LITERATURE REVIEW

Origin of Milk Fat

The hypothesis that the milk fat is formed in the mammary gland from the blood phospholipids was first put forward by Meigs et al (59) in 1919. The basis of their conclusion was the difference in the phospholipid content between the blood drawn from jugular blood (assumed to be the arterial sample) and the mammary vein (Kaufmann-Magne technique) of lactating cows. That this technique gave erroneous results was shown conclusively by Blackwood and Stirling (12) in 1932, who pressented evidence for a considerable hemoconcentration in the blood passing through the head region due to the removal of water by the salivary glands of cows. Subsequent workers using arterio-venous analyses of fat content have shown conclusively that a major portion of the milk fat of ruminants come from the blood triglycerides. For instance, Lintzel (49) found a removal 6-10 mg. of fat per 100 ml. of blood passing through lactating mammary glands of goats. The values obtained for lactating bovine mammary glands are as follows: 4.1 mg./100 ml. of blood (average of 244 determinations) by Graham, Jones and Kay (28), 3-52 mg./ 100 ml. of plasma by Maynard et al (56); 4-14 mg./;00 ml. of blood by Shaw and Petersen (84).

The total amount of blood traversing the mammary gland of a lactating cow was calculated from the calcium and phosphorous secreted in milk in 24 hours, by Shaw et al (86). It was calculated that 494 volumes of blood needs to go

through the gland for the secretion of one volume of milk. From this blood volume to milk ration, it was found that the fat removed by the gland from the blood accounted for about 93% of the fat secreted in milk.

Origin of Short-Chain Fatty Acids of Milk Fat

The precursors of the short chain fatty acids, which constitute nearly 10% by weight of the total fatty acids present in the milk fat of ruminants, has been the subject of intensive study during the last ten to fifteen years. Three possible methods have been discussed: 1) they may be formed by the degradation of long chain fatty acids in the mammary gland; 2) they may be synthesized from carbohydrates; 3) they may be synthesized from the volatile acids, mainly acetic acid, absorbed by the mammary gland from the blood.

The first theory that the short chain fatty acids are the degradation products of long chain unsaturated acids was advanced by Hilditch and collaborators (33) on the basis of the study of the glyceride pattern of the food, depot and milk fats. The glyceride structure suggested that the milk fats are formed from oleo-or linoleo- glycerides and that the short chain fatty acids are formed by a process of omega oxidation, followed simultaneously by a reduction of the oleic or linoleic acid part of the unsaturated glycerides. Further support for this theory are: 1) the position of the double bond in all the unsaturated acids containing ten carbon or more is always the same as in oleic acid viz.

between the ninth and the tenth carbon and 2) the inverse relationship observed between the short chain fatty acid and the unsaturated fatty acid contents of milk fats. Any treatment which causes the decrease of the short chain fatty acid content of milk fat, e.g. inanition, cod-liver oil feeding etc., is always accompanied by an increase in the proportion of the unsaturated fatty acids. Although circumstantial evidence in favor of this hypothesis is strong the lack of direct evidence of the occurrence of omega-oxidation of fatty acids in the mammary gland prevents the general acceptance of the theory.

The theory that the short chain fatty acids are formed from carbohydrate source is based entirely on the respiratory quotients obtained for lactating mammary glands (74). A value of higher than unity has been taken to signify the formation of fat from some carbohydrate source. The R. Q. is now generally admitted to be not decisive in the drawing of conclusions regarding the metabolic pathway and hence this theory has lately become more or less obsolete.

The theory that the lower fatty acids, mainly acetic, might be the precusors of the volatile fatty acids of milk fat gained considerable grounds ever since it was shown that the rumen of ruminants produce relatively large amounts of lower fatty acids (70) which are absorbed from the rumen (7).

Acetate Metabolism of the Rumenant Mammary Gland

Kaufmann and Thaw (42) showed, in 1945, that the volatile acids of milk fat were closely associated with the

dietary carbohydrates and suggested that the carbohydrates might be converted into the necessary precursors in the rumen by microbiological action. It had been shown previously by Shaw, Powell and Knoop (86) that the Reichert-Meisal number and Polenske number, which are an indication of the quantity of the volatile acids in the milk fat, do not depend upon the level of blood glucose. It was observed that in ketotic animals with marked hypoglycemia the R.M. value of milk fat did not decline as long as the cow was still on feed; conversely, the R. M. values of milk fat decreased considerably on fasting the animals, even for short periods of time (12 to 24 hours) while the glucose level in the blood was still the same. Mann and Shaw (53) then approached the problem in a different way. They attempted to prevent the decrease in R. M. value due to inanition by continuous abomasal feeding of goats and intravenous feeding of cows with various suspected precursors of short chain fatty acids of milk fat. The substances tried were glucose, glucose and "peptiease" (a protein hydrolysate), sodium oleate, sodium acetate and sodium butyrate. Excepting sodium butyrate all the other substances failed to prevent the lowering of R. M. values in spite of relatively heavy doses of the substances used. Sodium butyrate in the first trial not only maintained the R. M. value for a period of 36 hours but an actual increase in Polenske value was obtained. This significant finding, however, could not be repeated.

The presence of relatively large amounts of volatile fatty acids in the arterial blood of ruminants was shown by

McClymont (57). For cows the values ranged from 7 to 12 mg. per 100 ml. of blood or volatile acids expressed as acetic acid. McClymont (58) also found that the gland removes 2 to 6 mg. of acetate per 100 ml. of blood, which on a percentage basis represents 40 to 80 per cent of the arterial level. This relatively high value for acetate utilization by the mammary gland puts acetate as one of the major metabolites.

In a series of papers, Folley and French (22, 23, 24) described their work on the acetate metabolism of the mammary gland by the Warburg technique. They found that the R.Q. of ruminant mammary gland was invariably higher than unity when acetate was used as substrate, and less than unity with glucose. A non-ruminant mammary gland, on the other hand, gave an R.Q. less than unity with acetate and greater than unity with glucose. These observations of Folley and French show conclusively that the ruminant mammary glands are particularly adapted to the utilization of acetate for their metabolism and that acetate was being utilized for the syntheses of compounds relatively poorer in oxygen, e.g. fats or fatty acids. There might be some criticism of the conclusions drawn on the basis of R.Q. on tissue slices regarding the nature of metabolism, particularly when the tissue is as hard and lobular as the mammary gland, which makes the cutting of uniform sections very difficult. Secondly, the uneven and lacy sections obtained, no doubt due to the alveolar tissues, makes the determination of the thickness of

the sections difficult. Folley and French determined the thickness of liver slices cut under similar conditions and used this value for the thickness of the mammary gland tissue, a procedure the validity of which might be questioned, especially, when the thickness of the tissue had been observed to be necessarily less than 0.4 mm. thick and an increase in this thickness by as much as 0.2 mm. led to irregular and haphazard results. Milk present in the alveoli of the tissue slices is another complicating factor. However, the uniformity of the data obtained by these workers which could be satisfactorily reproduced perhaps makes these criticisms less important.

An *in vivo* study of the acetate metabolism was conducted on a goat by Popjak, French and Folley (71) using sodium acetate, labelled with C^{14} in the carboxyl group. It was found that nearly 80% of the injected acetate was oxidized to carbon dioxide in about 6 hours and the half-life of the body acetate was about 50 minutes. The rapidity with which acetate is utilized by the mammary gland can be gauged from the fact that 50% of the amount of radioactive carbon retained by the whole body appeared in milk in 6 hours. The half-life of the acetate in the formation of milk fatty acids appeared to be 4 hours. From the precursor-product relationship studies it could be concluded that plasma fatty acids are not the source of the volatile fatty acids of milk fat, neither is plasma cholesterol the source of milk cholesterol.

Cowie *et al* (16) perfused the oxytocin milked-out udder of a cow, with the perfusion apparatus of Peeters and

Hansart with blood containing labelled acetate. The control half was perfused with blood containing labelled sodium bicarbonate. By comparing the specific activities of the various chemical constituents of the udder tissue and milk it seemed clear that there was considerable oxidation of free acetate and the syntheses of the various volatile fatty acids (C_4-C_{14}) took place by a condensation of C_2 units. The latter conclusion was drawn from the fact that the specific activity of the fatty acids decreased with the increase in chain length. Acetate was incorporated in milk cholesterol and lactose also. The site of synthesis of milk cholesterol was the mammary gland itself. On the examination of the specific activities of the various fractions of glyceride fatty acids it could be seen that the concentration of C^{14} was 7-10 % greater in the gland fat than in the corresponding fractions in the milk fat, which could mean a considerable dilution of the milk secreted during the perfusion time with preformed milk not removed by one milking after a single oxytocin injection. A relatively rapid utilization of acetate by the gland for the syntheses of higher acids could also explain the results.

Dix, Petersen and Strauss (61) conducted a similar perfusion experiment using the same quantity of labelled acetate viz., 1 millicurie. These workers report activity in all fractions of glyceride fatty acids, cholesterol, casein, non-casein protein, non-protein-nitrogen fraction, citric acid, lactose etc. On comparing the specific activities of the water soluble volatile acid of the gland fat, the fraction giving

the maximum activity in their data, with the activity of the corresponding fraction of Cowie et al (16). Secondly, while Cowie et al obtained for the milk fatty acid fractions a specific activity value of 7-10% of the value obtained for the corresponding fractions obtained from the udder tissue, Mix et al obtained only 0.7% of the glandular activity in milk fat fractions. This could only mean that Mix et al had practically no milk synthesis in the gland. Counts as low as 0.18 per minute per milligram of carbon are reported which are too low for the drawing of reliable conclusions.

From a study of the intravenous injection of labelled acetate to the intact cow, Kleiber et al (45) found that of the carbonyl carbon of acetate, 1% is obtained in lactose, 1% in casein and 9% in milk fat while the corresponding values from methyl carbon were 2,2 and 12% for lactose, casein and milk fat respectively. This study also gave evidence for the more or less direct condensation of the 2 carbon units of acetate to form fatty acids, while a substantial portion of the carbon atoms of acetate ending in lactose and casein pass through the carbonate pool of the body of the cow.

The milk fat obtained from the goat injected with labelled acetate in the study of Popjak, French and Folley (71) was subjected to further detailed study by Popjak, French, Hunter and Martin (72). They divided the fat into two fractions: 1) obtained upto 12 hours after injection and 2) that obtained between 12 to 48 hours. The fatty acids obtained from each of these milk fats were fractionated and the

specific activities of C^{14} determined. In the first fraction the highest specific activity was obtained in the steam volatile water insoluble fatty acids the soluble acids coming next. Around 12 hours after injections there was a crossing over of the specific activities so that at 23 hours the order of the specific activities were:non volatile solid acids$\rangle$insoluble volative acids$\rangle$soluble volatile acids$\rangle$ non volatile liquid acids. Specific activity determinations of individual acids showed that acetate incorporation into fatty acids increased with chain length upto and including C^{10} acid (capric) in the first twelve hours while later on it increased upto myristic and palmitic acids. Butyric acid had an activity lower than any acid in both periods; upto lauric in the first twelve hours and later on lower than palmitic. Butyric was found to be labelled equally in the carboxyl carbon (or carbon 1) and carbon 3, carbons 2 and 4 being inactive. A stepwise degradation of caproic acid showed that this acid was formed by the condensation of a C_2 unit with butyric acid since the activity of carbon 3 to 6 was found to be the same as butyric acid. Surprisingly, the specific activity of the carboxyl carbon was 2.5 times the activity of C_3 and C_5. This was explained by the authors by proposing that the butyric acid synthesized from acetate was diluted 2.5 times by butyric acid from some other source. This supposition also required that the source or precursor of butyric acid be a 4-carbon compound. Shaw and Knodt (83) had previously suggested beta-

hydroxybutyric acid as a possible precursor for butyric acid of the milk fat.

Beta-hydroxybutyric Acid Metabolism of the Mammary Gland

Three chemical compounds, beta-hydroxybutyric acid, acetoacetic acid and acetone are generally grouped under the term, "ketone" or "acetone" bodies. It is generally agreed that the ketone bodies are formed mainly from fats. The formation of acetoacetic acid was first explained by Dakin (17) on the basis of Knoop's (46) work on phenyl substituted fatty acids. He postulated the beta-oxidation of fatty acids, according to which fatty acids are broken down by the sucessive oxidation and splitting of two carbon atoms, from the carboxyl carbon of the fatty acids to form acetic acid until a four carbon compound remained which was also oxidized at the beta-carbon but was not split. This theory provided for one molecule of the four carbon compound, acetoacetic acid, for each molecule of fatty acid oxidized. The formation of proportionately more acetoacetic acid from longer chain acids (41), lower consumption of oxygen than required by the theory and the failure to identify the presence of acetic acid in the liver led Hurtley (37) to propose his theory of multiple-alternate oxidation. This theory explained the oxidation of fatty acids by assuming simultaneous oxidation at alternate carbon atoms with splitting of the molecule into units of four carbon atoms. As this theory also did not explain all the observations e.g. the production of ketone bodies from valeric acid observed by Jowett and Quastel

(40, 41) and McKay et al (52), the increased production of ketone bodies with the use of acetate obtained by McKay et al (51), McKay and coworkers (52) proposed the currently accepted theory of beta-oxidation followed by the condensation of two acetic acid molecules to form acetoacetic acid. Confirmation of this theory came from the tracer studies of Weinhouse, Medes and Floyd (95) and Buchanan and coworkers (15). The oxidation of carboxy-labelled n-octanoic acid by liver tissue resulted in the production of acetoacetic acid in which both the carboxyl and carbonyl groups, were labelled an observation explainable only by the condensation of two carbon fractions to form acetoacetic acid.

Acetone bodies although considered by early physiologists to be abnormal products due to incomplete combustion of fatty acids are now recognized as normal metabolites of most of the organs and tissues of the body (67) Barnes et al (8) showed by the perfusion of an isolated heart-lung that the extent of beta-hydroxy butyric acid utilization was sufficient to account for 80% of the oxygen consumed. Nick and Drury (96) found the utilization of beta hydroxybutyric acid to depend upon its concentration in blood upto the point where it accounted for about 90% of the oxygen consumed.

The utilization of beta hydroxybutyric acid by the lactating bovine mammary gland was first shown by Shaw and Knodt (83). On comparing the metabolism of the mammary gland of normal cows and cows with ketosis Shaw (81) found that the latter used over 100% of the beta-hydroxy-butyric acid metabolized by the gland of the normal cow. In contrast to

the finding of Wick and Drury (96) Shaw did not find that the utilization of beta-hydroxybutyric acid stopped with the quantity accounting for 90% of the oxygen consumed, assuming complete oxidation. This difference can be considered within the limits of experimental error. The work of Shaw and coworkers also showed that only the beta-hydroxybutyric acid fraction of the ketone bodies of blood was utilized by the mammary gland.

In order to study the behavior of the gland towards beta-hydroxybutyric acid in the presence of glucose, Shaw and Petersen (85) perfused bovine mammary glands with blood containing varying amounts of beta-hydroxybutyric acid. Nine perfusion trials confirmed the large uptake of beta-hydroxybutyric acid and the non-utilization of acetone and acetoacetic acid. In one detailed balance study the beta-hydroxybutyric acid utilized accounted for 88% of oxygen consumed. That the increased utilization of beta-hydroxy-butyric acid was not due to the non availability of glucose was also brought out in this study.

Utilization of Acetate

Acetate utilization by the perfused mammary gland was studied in four perfusion trials. The apparatus has been described in a previous section. Pumps were adjusted to give a pulsatile flow at a systolic pressure of 100-110 mm. of mercury. One half of the gland was perfused, while the other half served as a control. Using a 20% solution, the acetic acid content of the blood was increased to any desirable level at the beginning of the perfusion.

The volatile fatty acids of the blood were determined by the method Scarisbrick (79) using Markham's (54) apparatus and were expressed as acetic acid in mg. per 100 ml. of blood. The volatile fatty acids of the udder tissue were determined by macerating the gland, rendering the macerate slightly alkaline, boiling and using the filtrate for "acetate" determination by the method used for blood.

The amount of acetate removed from the blood by the gland was determined by the analyses of acetate at the beginning and after the termination of the perfusion. The difference in the contents of the volatile fatty acids of the perfused and the control halves of the udder was assumed to be the non-metabolized acetate. Subtracting the non-metabolized acetate content from the amount of acetate absorbed from the blood, the amount of acetate metabolized by the gland was obtained and expressed as milligrams of acetate utilized by 100 grams of the glandular tissue per

100 ml. of blood passing through it. The data are given in table 18.

A constant utilization of acetate per unit weight of the tissue per unit volume of blood flowing through it was noticed.

A further study was made to determine whether the bovine mammary gland shows any preference between the two substrates, acetate and beta-hydroxybutyrate. It was felt that the arteriovenous differences of these substrates would give more reliable data than the analyses of blood at the beginning and the end of the perfusion, for the following reasons: 1) the arteriovenous difference in metabolites represents a truer picture of the metabolism of the gland, after equilibrium has been established between the blood and the glandular tissue, 2) an accurate assessment of the metabolite in the perfused gland is difficult, as the proportion of the secretory tissue in the gland as differentiated from the adipose tissue cannot be accurately determined because of its rather uneven distribution, and 3) the presence of milk in the alveoli is another complicating factor.

The perfusion apparatus described in a previous section was used with the following modifications: 1) the gland was enclosed in a glass carboy and 2) a rubber lined centrifugal pump (obtained from Schaar and Co.) was used for the delivery of the arterial blood. The blood flow, thus, was non-pulsatile.

Beta-hydroxybutyric acid was added before introducing the blood into the apparatus. At least one complete passage of the entire amount of blood used through the gland was allowed before taking the first set of arteriovenous samples. Two subsequent sets of samples were taken, one set for each passage. The beta-hydroxybutyric acid in the blood was determined by the method of Weichselbaum and Somogyi (94). The total quantity of acetone bodies determined by this method was expressed as milligrams of beta-hydroxybutyric acid per 100 milliliters of blood.

After the third set of arteriovenous samples was drawn sodium acetate was added in sufficient amounts to give approximately 20-25 milligrams per 100 milliliters of blood. One complete passage was allowed for the attainment of equilibrium between the acetate of the blood and the acetate being metabolized in the gland. Two to three sets of arteriovenous samples were taken in subsequent passages of blood. Acetic acid was determined in these blood samples by the method of Scarisbrick as before. Usually, analyses could be made only on the first two sets of samples as the blood samples for acetate determination deteriorated rather quickly. The addition of sodium fluoride or thymol as preservatives interfered with the analysis. The beta-hydroxybutyric acid content was determined in all the samples.

A preliminary study, in which the beta-hydroxybutyric acid was not analysed, showed a constant acetate utilization by a lactating mammary gland after 45 minutes of perfusion. The data are presented in table 19.

Table 18

The Utilization of Acetate by the Perfused Mammary Gland.

	PERFUSION NO.			
	6	7	9	10
Duration of perfusion (min)	120	95	50	120
Vol. of blood used (liters)	6	6	3	7
Weight of the perfused gland (grams)	4,200	10,300	6,170	6,700
No. of times the blood passed through the gland (calculated)	3	6.8	5.4	5.9
Acetate level of blood (mg%): Initial	33.8	54.9	62.8	37.4
Final	12.0	0.7	13.9	15.0
Acetate taken up by the gland (grams)	1.31	3.252	1.467	3.568
Acetate level in the gland (mg%) Control		9.8	6.4	13.7
Perfused		4.1	12.2	16.8
Acetate gained by the perfused gland (gram)		-0.588	0.357	0.208
Acetate metabolized by the gland (gram)	1.31	3.84	1.11	4.41
Acetate utilized (mg /100g tissue/ 100 ml of blood)	0.17	0.09	0.10	0.16

Table 19

Utilization of Acetate by the Mammary Gland after one Hour of Perfusion (perfusion 74)

Perfusion time (min)	Rate of blood flow (ml/min)	Acetic acid in blood (mg %)[1]		A-V difference (mg %)
		Arterial	Venous	
0	-			
10	500			
25	440			
45	500			
65	-	32.52	26.64	5.88
90	-	23.28	17.28	6.00

[1] 2g Acetate was added at 46 minutes perfusion time.

The data show that the gland was still functional after 45 minutes and that this procedure of study might be expected to give consistant results. Some preliminary studies had shown that unless sufficient blood passed through the gland, equilibrium between the metabolites of the blood and gland were not obtained. Also, a dry gland showed no withdrawl of acetate from the blood after the initial process of diffusion had equilibrated. These observations indicated the reliability of the procedure.

The data on three perfusion studies are presented in tables 20, 21 and 22. They show more or less conclusively that the availability of acetate inhibited the utilization of beta-hydroxiybutyric acid by the mammary gland.

Table 20

The Effect of the Addition of Acetate on the utilization of Beta-hydroxybutyric Acid (Perfusion 76).

Perfusion time (min)	Rate of blood flow (ml/min)	Beta-hydroxybutyric acid in blood (mg %)			Acetic acid in blood (mg %)		
		Arterial	Venous	A-V diff.	Arterial	Venous	A-V diff.
0	-						
11	250						
16	520						
28	565	24.86	20.56	4.30			
44	600	21.92	16.32	5.60			
63	600	9.74	4.02	5.72			
100	630	12.39	12.22	0.17	15.72	9.12	6.60
121	650	15.35	12.91	2.44	12.00	6.96	5.04
136	650	9.26	8.20	1.60	-	-	-

[1] 2 g. sodium acetate added at 80 minutes perfusion time.

Table 21

The Effect of the Addition of Acetate on the Utilization of Beta-hydroxybutyric Acid (Perfusion 80)

Perfusion time (min)	Rate of blood flow (ml/min)	Beta-hydroxybutyric acid in blood (mg %)			Acetic acid in blood[1] (mg %)		
		Arterial	Venous	A-V diff.	Arterial	Venous	A-V diff.
0	-						
5	520						
22	-	49.76	40.83	8.93			
35	450	39.24	32.54	6.70			
46	-	32.86	30.00	2.86			
67	485	31.58	31.58	0.00	14.40	8.52	5.98
80	-	30.31	31.20	0.89	16.44	11.52	4.92

[1] 2 g. sodium acetate added at 50 minutes perfusion time.

Table 22

The Effect of the Addition of Acetate on the Utilization of Beta-hydroxybutyric Acid (Perfusion 82)

Perfusion time (min)	Rate of blood flow (ml/min)	Beta-hydroxybutyric acid in blood (mg %)			Acetic acid in blood[1] (mg %)		
		Arterial	Venous	A-V diff.	Arterial	Venous	A-V diff.
0	-						
3	800						
23	950	56.72	54.28	2.44			
38	1,050	53.40	49.78	3.62			
50	1,100	47.37	43.88	3.49			
65	1,200	41.47	40.72	0.75	9.96	4.32	5.64
71	1,300	42.24	37.21	5.03	1.20	-	-

[1] 2 g. sodium acetate added at 52 minutes perfusion time.

DISCUSSION

The four perfusions, in which the total amount of acetate utilized by the gland were studied, were done in the early stages of the commencement of this study. The rates of blood flow obtained were poor or an initial high rate of flow declined markedly during the course of the perfusion. The rates of blood flow obtained in perfusions 6 and 7 are given in tables 1 and 4 respectively and in perfusions 9 and 10 in table 2. In spite of the wide variations in the rate of blood flow, the level of acetate in the blood and the weights of the mammary gland, a relatively constant acetate utilization was obtained per unit weight of the gland per unit volume of blood going through the gland. This showed that more than a simple matter of diffusion was involved and also that the capacity of the gland to metabolize acetate was fixed.

The amount of acetate metabolized by the gland was obtained by subtracting the volatile fatty acids gained by the gland from the quantity of the volatile fatty acids removed from the blood. This assumed that the volatile acids remaining in the gland was the acetate removed from the blood which was not metabolized. The distillation procedure of Fearisbrick determines all the free volatile acids, thus butyric acid or any higher volatile acids synthesized from acetate but not incorporated into the glycerides would be included in these data as non metabolized acetate.

The work of Folley, Popjak and coworkers (16, 71) and

Kleiber and his group (45) show that there are many pathways by which acetate is metabolized. It is seen from the results of these workers that acetate is used by the mammary gland for the syntheses of cholesterol, lactose and casein in addition to the fatty acids in the triglycerides of milk. Cowie et al observed nearly 40% incorporation of the labelled acetate in the fatty acids with significant amounts in lactose and cholesterol. Although no estimation could be made of the amount of acetate oxidized to carbon dioxide the rapidity with which this reaction takes place in the mammary gland was shown by the high specific activity of the blood carbon dioxide at the beginning of the perfusion. The specific activity of the blood carbon dioxide at zero time was 3 to 7 times the value obtained subsequently. These data and the data on intact animals indicate that the combustion of acetate is one of the most predominant reactions of tissue metabolism. The work of Kleiber et al (45) also indicates that there are metabolic pathways for the syntheses of lactose and casein from acetate other than through the oxidation to carbon dioxide and its subsequent fixation. The significat difference in the incorporation of carboxyl- and methyl-carbon atoms of the acetate in these compounds also suggests different mechanisms for the metabolism of the two different carbon atoms of acetate. Fatty acids on the other hand appeared to be formed more or less exclusively by a direct condensation of acetate molecules or at least some two-carbon units. The extraordinary complexity of acetate metabolism in the mammary gland is thus very obvious.

The acetate utilization in these four perfusion experiments ranged from 0.09 to 0.17 milligram per 100 milliliter of blood per 100 gram of the glandular tissue with a mean value of 0.13. Taking into account the fact that the free fatty acids synthesized from acetate is not included in these values, these figures represent the minimum amount of acetate metabolized. Whichever the pathway of metabolism the acetate followed, a major portion of it had gone farther than condensation to form longer chain free fatty acids. If longer chain acids were formed they were incorporated into the glyceride molecules. The considerable excess of the acetate metabolized over the fraction assumed to be acetate and remaining unmetabolized would also tend to show that metabolic reactions did take place in the perfused mammary gland to an appreciable extent.

The study of the competition between beta-hydroxybutyric acid and acetic acid show that the gland preferred acetate to the former. In the perfusion described last (No. 82) a more or less constant utilization of beta-hydroxybutyric acid was obtained in the first three arteriovenous analyses prior to the addition of acetate. The absorption of beta-hydroxybutyric acid was suppressed on the addition of acetate (shown by the arteriovenous analyses at 65 minutes). By the time the next A-V samples were drawn all the acetate was utilized and the utilization of beta-hydroxybutyric acid began again. This shows in the first place that the gland was still active and that as long as acetate was available to the gland beta-hydroxybutyric acid was not utilized.

From the values presented it can be seen (except some of figures in perfusion 76) that the arterial level of beta-hydroxybutyric acid was approximately the same as the venous level of the previous set of samples when this compound was being utilized by the gland. On the addition of acetate to the blood the values of beta hydroxybutryric acid continued to be of the same order, further confirming the non-utilization of this substrate. For instance, in perfusion 82 the initial value of 56.72 milligram per cent steadily declined to 43.88 milligram in 50 minutes. For the next 20 minutes while acetate was being utilized the value of beta hydroxybutyric acid remained nearly the same and when most of the acetate of the blood had been depleted the beta hydroxybutyric acid was utilized again.

The arteriovenous difference in acetic acid in all the four perfusions ranged from 4.92 mg. to 6.6. mg. per 100 ml. of blood, most of the values being between 5-6 milligrams. Such constancey of values are not likely to be fortuitous. These values compare very well with the arteriovenous differences obtained by McClyment (58) on live cows. The values obtained for the beta-hydroxybutyric acid in these studies were more variable and in general tended to be slightly higher than the values obtained by Shaw (81) in cows with ketosis. However, the figures in all the perfusions except the first two in perfusion 80 are close enough to those obtained in intact cows to preclude the possiblility of abnormal metabolism in the perfused mammary gland.

A preference for acetate over beta hydroxybutyric acid by the mammary gland would suggest that these two metabolites are used by the gland for similar, if not identical, metabolic functions. Contrariwise, the independence of beta-hydroxybutyric acid utilization to the glucose level (82) can be taken to mean that these two metabolites are used by the mammary gland for different functions. Shaw and Petersen (84) calculated that almost all of the glucose taken up by the gland was necessary for lactose synthesis so that very little would be available for oxidation for energy purposes. Assuming this calculation to be correct, the energy for the various synthetic processes of the gland must be obtained by the combustion of some other metabolite. Acetate is undoubtedly one such metabolite and if the identity of the metabolic function of beta hydroxybutyrate with acetate is is assumed, beta hydroxybutyrate would be a second metabolite. Shaw (81) calculated that in the normal gland 37% of the oxygen consumed can be accounted by assuming complete oxidation of beta-hydroxybutyric acid while practically all of the oxygen would be required for the oxidation of this metabolite in ketotic cows. Such calculations of course do not necessarily mean that these reactions do take place but only suggest a possibility.

Since acetate has been definitely shown to be used for the syntheses of longer chain acids of milk fat, it is possible that beta hydroxybutyric acid likewise is used for such syntheses. Added support for this assumption comes from the data of Popjak et al (72) on the specific activities

of individual carbon atoms of caproic acid of the milk fat obtained from goat injected intravenously with labelled acetate. These workers calculated that the butyric acid formed from acetate condensation was diluted 2.5 times with some other source of butyric acid and suggested beta-hydroxybutyric acid as a possible precursor, this being the only known 4 carbon metabolite of the mammary gland.

The role of beta hydroxybutyric acid as a precursor for the lower fatty acids of milk fat was considered by Shaw and Knodt (83) but was abandoned (86) because of the marked lowering of Reichert-Meisal and Polenske values of cows with ketosis. It was argued that since the volatile fatty acid content of milk fat decreases, inspite of the greater availability and utilization of beta hydroxybutyric acid in ketosis, this compound cannot be the precursor of these fatty acids.

If the indication from the data presented here that acetateand beta hydroxybutyrate are used for identical or at least similar bio-chemical reactions have any validity, then the acetate appears to be used with greater facility. The unavailability of acetate to the gland would then change the course of the metabolism to the usage of beta-hydroxy-butyric acid. The relative amounts of acetate and beta-hydroxybutyrate taken up by the gland under normal conditions is difficult to determine, as the acetate absorbed by the gland under normal conditions is difficult to determine, as the acetate absorbed by the gland has been shown to

vary with its arterial level and the arterial level is known to fluctuate considerably with time after feeding (57, 58). The probability of using both these compounds for the synthetic processes under normal conditions are strong but conclusive evidence that such is the case will have to await tracer studies with beta-hydroxybutyric acid.

CONCLUSIONS

The perfusion of bovine mammary gland with blood presents certain difficulties which can be overcome in the perfusion of the organs of small animals. Heparinizing the animal before bleeding, the slaughter of the animal with little excitement by the use of anesthetics, perfusion of the gland *in situ* etc. are somewhat prohibitive in bovine udder perfusions in view of the cost involved. These precautions if taken would undoubtedly, result in better perfusions.

Barring all these factors, care of the nature of the arterial blood flow, the blood pressure and the mode of suspension had definite effects on the perfusion rate. Thorough filtration of the blood before the perfusion was very essential as a certain amount clotting almost always takes palce when handling large quantities of blood. Perfusion of a lung prior to the perfusion of the mammary gland appeared to have a beneficial effect in a few perfusions.

A hitherto unexplainable vasoconstrictive effect due to the oxygenation of blood with pure oxygen was observed. 5 per cent carbondioxide in oxygen, as well as the addition of chloral hydrate prevents the vasoconstriction. The latter confirmed the involvement of the nerves associated with the production of vasoconstriction, suggested by the rapidity of the changes of rates of blood flow.

The metabolic activity of the perfused gland undoubtedly would be closer to normal with shorter time-lapse

between the shedding of the blood of the animal and the establishment of the blood circulation with the apparatus.

Using the perfusion technique the influence of the presence of acetate in the blood on the uptake of beta-hydroxybutyric acid was determined. It was seen that the presence of acetate inhibits the uptake of the ketone body even when the latter was present in rather high levels. Such a competition between these two important metabolites indicates an identical or at least a similar main metabolic pathway, in which the acetate is one step ahead of the betahydroxybutyric acid. The possibility of its being used for conversion to butyric is suspected but conclusive proof of such a reaction can be obtained only by the use of labelled beta-hydroxybutyric acid.

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