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A MICRO METHOD FOR MEASURING CHOLESTEROL UPTAKE

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School of Aerospace Medicine
Aerospace Medical Division (AFSC)
United States Air Force
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Project No. 7753, Task No. 775301

School of Aerospace Medicine, Brooks AF Base, Tex. SAM-TDR-62-84. A MICRO METHOD FOR MEASURING CHOLESTEROL UPTAKE. Aug. 62, 6 pp. incl. illus.

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FOREWORD

This report was prepared by the following personnel in the Biokinetics Branch:

DALE A. CLARK, Ph.D.
CARRIE B. HAVEN, B.S.

The authors are grateful for the skillful assistance of Walter O. Baentsch, Instrument Section, who fabricated the glassware described herein.

ABSTRACT

A method has been devised by which the cholesterol uptake of serum can be measured on volumes as small as 1.2 ml. Equipment by which this procedure can be carried out is described. Key to the use of the small volumes is a flexible plastic incubation chamber and a unit in which filtration, centrifugation, and pipetting can be carried out without the necessity of transferring the filtrate to another vessel. The equipment is useful for other laboratory procedures which involve filtration of small volumes. The procedure used in micro measurement of cholesterol uptake is described.

This technical documentary report has been reviewed and is approved.

Robert B. Payne

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A MICRO METHOD FOR MEASURING CHOLESTEROL UPTAKE

1. INTRODUCTION

An earlier report (1) from this laboratory described a method for incubating serum with solid cholesterol and measuring the cholesterol uptake (the increase in cholesterol concentration in the serum after the incubation). The method utilized 15 ml. test tubes as incubation vessels and an ordinary laboratory funnel fitted with a filter paper as a filtration device. To provide an adequate volume of filtrate after the incubation, it was necessary to use relatively large volumes of serum in each incubation tube. Some serum was lost by absorption to the filter paper and to the solid particles of cholesterol. The latter loss was particularly noticeable in those tubes to which the larger quantities of cholesterol had been added. The total volume required for a determination of cholesterol uptake was 25 cc. of serum, obtainable from approximately 60 cc. of whole blood. Obviously, this volume of blood is too large for use in studies on small laboratory animals or human beings when the measurements must be repeated at intervals of a few hours.

The limitations of the macro method led to the development of a micro method for measuring cholesterol uptake. In developing the micro method, the two major problems were (a) to decrease the volume of serum required, and (b) to separate the serum and residual cholesterol without concentration of the filtrate by evaporation and with minimal loss in filter paper, pipets, etc. The requirement for use of small volumes of serum without danger of concentration due to evaporation ruled out the possibility of vacuum filtration to separate the cholesterol from the serum after incubation. Centrifugation alone was inadequate because small cholesterol particles sometimes floated on the serum. Filtration was therefore required. Transfer of the incubated serum from incubation chamber to centrifuge tube and thence to filtration apparatus entailed unacceptable losses in volume. Therefore a unit was devised in which centrifugation, filtration, and pipetting could be accomplished without transfer of the serum from one vessel to another. Conducting the incubation itself in the same vessel in which centrifugation and filtration were to be accomplished proved impracticable. The equipment now used therefore includes two units: (a) the filtration unit and (b) the incubation chamber.

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2. APPARATUS

The design of one of the filtration units is illustrated in figure 1. This apparatus is essentially a U tube in which the straight arm has been cut to provide a flat surface on the upper end that serves as a seat for the small filter paper disc. A short length of plastic tubing attached to the upper end serves as the connecting tubing into which the square cut end of the pre-filter is inserted. This connecting tubing keeps the filter paper disc compressed between the flat surfaces of the pre-filter and the top of the filtration unit. This arrangement is similar to the filter described by Pregl and Grant (2). On the other arm of the U-shaped filtration unit is a bulb (capacity approximately 0.3 ml.) in which to accumulate the filtrate. The outlet from the bulb is the pipet, which is simply a continuation of the capillary tubing drawn into a delivery tip and calibrated. The two sides of the U are drawn closely together so that the whole unit can fit inside the metal centrifuge shield which accommodates ordinary 15 ml. centrifuge tubes and be centrifuged in an ordinary laboratory centrifuge such as an International model V or SVB, equipped with a suitable head and

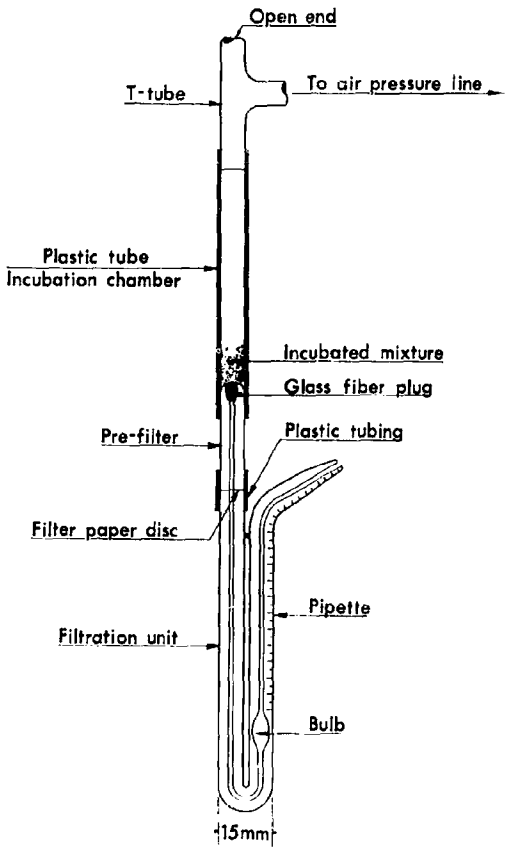

FIGURE 1

Apparatus assembled for filtration of incubation mixture.

trunnion rings. After the entire unit is assembled, filtration and centrifugation can be accomplished in one step. After separation of the fluid from the residue, the filtrate collects in the bulb and can be expelled via the outlet tube, which has been calibrated as a pipet. Thus, measured volumes of the filtrate can be dispensed directly without loss due to transfer operations.

The most satisfactory incubation chamber consists of a closed ring of plastic tubing, as illustrated in figure 2. An 8-cm. length of plastic tubing with internal diameter of 5 mm. is charged with cholesterol and serum, and bent into a circle. The circle is closed by joining the ends together with a ½-inch piece of stiff polyethylene tubing having an outside diameter of 5 mm. and an internal diameter of 3 mm. This incubation chamber is rotated by a suitable mechanical apparatus. As the unit rotates, the cholesterol and serum mixture flows along at the lowest point of the loop. This motion continuously agitates the mixture and repeatedly wets the inside walls of the loop, as well as any adhering cholesterol crystals. There is no possibility for some of the cholesterol crystals to escape contact with the serum.

The rotating device is pictured in figure 3. A small electric motor rotates the large disc at a speed of 15 r.p.m. The ring incubation chambers hanging on the pegs projecting from the large disc turn at a speed of 5 to 8 r.p.m. Their rate of rotation is a function of the ratio of the circumference of the peg to the inner circumference of the ring incubation chamber. One can vary the speed of rotation of the incubation chambers by varying the diameter of the pegs. As the circumference of the peg is increased, the speed of rotation of the incubation chamber also is increased except, of course, that the speed of rotation of the disc is the limiting speed. Too rapid rotation of the chambers, however, leads to poor mixing of serum and cholesterol. In use, the rotating disc is placed inside an oven or incubator at the desired temperature to maintain continuous rotation of the incubation chambers for the desired time.

An integral part of the use of the plastic tubing incubation unit is a short length of capillary tubing, the "pre-filter" unit, used to accomplish filtration. One end of this pre-filter unit is cut off square for close contact with the filter paper disc when the flat ends of the pre-filter and the filtration unit are butted together with the filter paper disc between (see figure 1). The other end of this capillary pre-filter unit is tapered to provide ready attachment of the plastic tubing incubation unit. The bore of the capillary tubing has been enlarged at this end as shown in figure 2 to make a cup into which a small piece of glass fiber filter paper is inserted before the

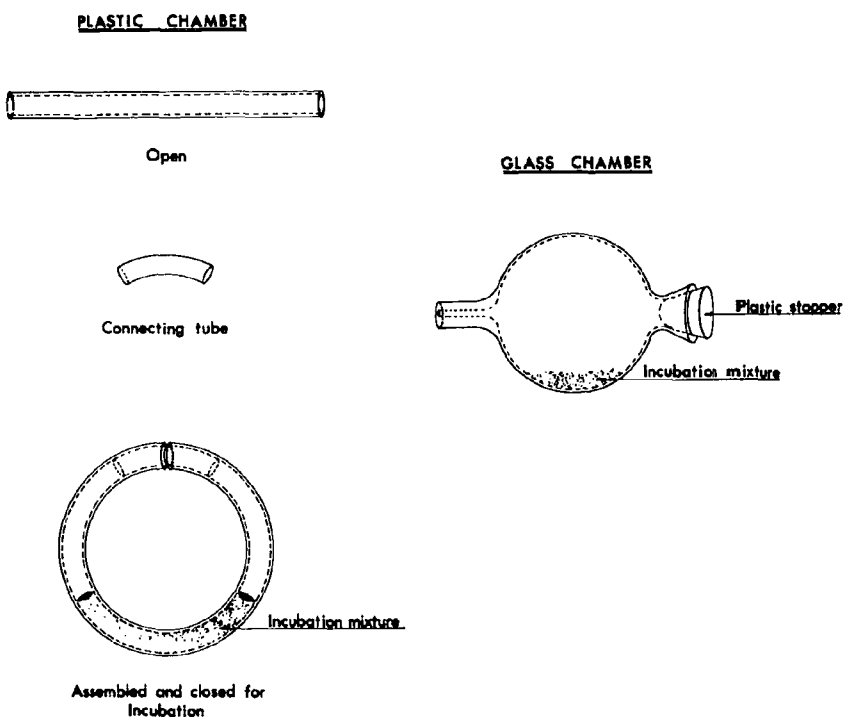

FIGURE 2

Incubation chambers for micro method of measuring cholesterol uptake.

incubation unit is attached. This "pre-filter" screens out the cholesterol crystals and prevents their accumulation in the bore of the capillary tubing. Experience in choosing the right amount of glass-fiber filter paper to insert into the cup is indispensable. Too large a piece is apt to become dislodged; too small a piece fails to keep the cholesterol crystals out of the capillary tube. With experience, however, satisfactory results can be obtained routinely.

For use in an incubation experiment, the pre-filter is assembled with the filter paper disc and attached to the filtration unit as shown in figure 1. After incubation of the serum with cholesterol in the plastic ring chamber, the ends of the incubation chamber are disengaged from the connecting piece and one end is inserted over the free end of the capillary pre-filter unit. Filtration can then be accomplished by centrifugation of the entire apparatus. However, a piece of glass tubing must be inserted over the outside of the plastic chamber to prevent its bending when centrifugal force is applied in the centrifuge. It has been found more convenient to accomplish filtration by the use of air pressure from an ordinary rubber squeeze bulb or an air pressure line. A T tube is inserted in the air line, as shown in figure 1. When the air pressure is turned on, finger pressure on the open end of the T can be used to control the air pressure on the fluid at the filter and, hence, the rate of filtration.

An alternate design of the filtration unit is simply a length of capillary tubing, one end of which has been cut off square and the other end of which has been drawn out to serve as

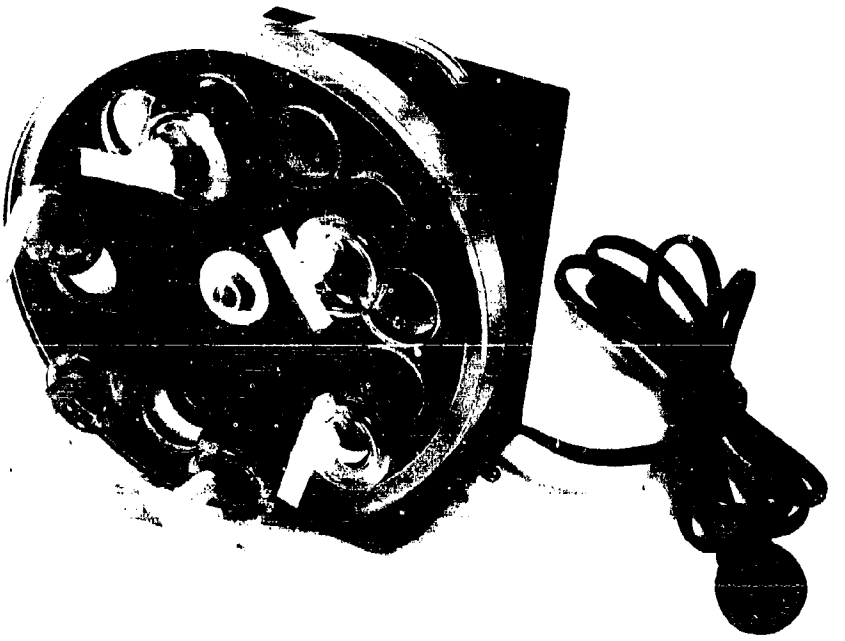

FIGURE 3

Photograph of rotating disc apparatus with plastic incubation chambers in place.

a pipet. This unit is connected to the pre-filter unit, in place of the U-shaped filtration unit. The assembled apparatus is supported in an upright position, and filtration occurs under the force of gravity combined with the effect of capillary attraction. Frequently this method of filtration is almost as good as the use of pressure because there is less tendency of the crystals to pack around the small plug in the upper end of the pre-filter unit. The long, straight capillary tube is calibrated for use as a pipet so that dispensing of measured volumes can be accomplished without transfer to intermediate receptacles. In practice, the use of this apparatus is complicated by the fact that frequently air bubbles are trapped within the fluid column in the long capillary. Since there is no bulb in which bubbles can rise to the top and be expelled, it is difficult to achieve a continuous fluid column in the long capillaries. To get rid of the bubbles, the fluid must be expelled from the capillary and drawn up again.

The first incubation chamber designed is illustrated in figure 2. It is essentially a small glass chamber in the form of a bulb with a capillary outlet on one end and a relatively large opening on the other end for charging the chamber. The delivery end of the capillary outlet has been cut off square in order to seat tightly against the filter paper disc and the top of the filtration unit, in place of the pre-filter unit described above. In actual use the chamber would be charged with serum and solid cholesterol, stoppered with a plastic stopper, and placed in a suitable mechanical apparatus to rotate the chamber gently at the desired temperature for a period of 4 hours. After incubation, the chamber would be attached to the filtration unit and the serum

filtered as described above. Unfortunately, many cholesterol crystals stuck to the wall above the level of the liquid phase and escaped contact with the serum. Therefore the plastic ring chambers described above were developed.

Although the glass incubation chamber proved unsatisfactory for measuring cholesterol uptakes, it has obvious advantages for many micro laboratory procedures. The unit is simple in design, can be dismantled easily for cleaning, and, above all, affords the possibility of providing a reaction chamber which can be connected to the filtration unit for filtration or centrifugation without the loss of sample in transferring from one vessel to another. Retention of fluid in the tiny filter paper disc is minimal. The usefulness of this apparatus is apparent in applications involving preparation of small amounts of precipitate and separation of the filtrate for subsequent analysis. The increasing use of micro laboratory technics suggests widespread applicability of this apparatus, as in micro methods for deproteinizing serum and analyzing the filtrate (3, 4).

3. METHOD

The routine procedure for micro determination of cholesterol uptake utilizes the plastic tubing incubation chamber. The chambers are charged with the desired amounts of cholesterol and of serum, as shown in table I, which also shows the control samples used. The ends of the chambers are joined by the plastic connecting link, and the resulting circular chambers are placed on pegs on the rotating disc and incubated at 37°C. for 4 hours. The filtration unit, filter paper disc, and pre-filter unit are assembled as shown in figure 1. At the completion of the incubation period, the closed incubation chamber is opened, one end is attached to the capillary pre-filter unit, and the other end to the pressure line, as indicated in figure 1. Gentle air pressure is used to effect filtration. The volume of filtrate is usually not less than 80 μl. when 0.2 ml. serum is used for the incubation. This filtrate is analyzed for cholesterol by the procedure of Rosenthal et al. (5) and for lipid phosphorus by the method of Bartlett (6).

4. DISCUSSION

A comparison of the results of measurement of cholesterol uptake by the micro and macro methods is appropriate. It must be emphasized that the cholesterol uptake measured with either procedure is probably the net difference between the cholesterol solubilized and the lipoprotein denatured or adsorbed during the incubation (7). Since the macro and micro procedures utilize different apparatus, it is conceivable that they may give different cholesterol uptakes with some serum samples. A comparison of the uptake measured on two serum samples by both macro and micro procedures is presented in table II. While there

TABLE I

Tube contents and handling for micro measurement of cholesterol uptake

Tube	Volume of serum (ml.)	Cholesterol added (mg.)	Cholesterol added (mg./ml. serum)	Incubated	Filtered
Controls					
Nonincubated	0.2	0	0	No	No
Incubated	0.2	0	0	Yes	Yes
Tubes					
A	0.2	8	40	Yes	Yes
B	0.2	16	80	Yes	Yes
C	0.2	24	120	Yes	Yes
D	0.2	32	160	Yes	Yes
E	0.2	40	200	Yes	Yes

TABLE II

Cholesterol uptake measured in two different serum samples by both macro and micro methods

Tube	Cholesterol added (mg.)	Cholesterol concentration							
		Serum A				Serum B			
		In filtrate		Change from control		In filtrate		Change from control	
		Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro
		(mg./100 ml.)							
Controls									
Nonincubated	0	221	221	—	—	254	268	—	—
Incubated	0	228	229	—	—	—	273	—	—
Tubes									
A	40	267	251	38	23	272	297	18	26
B	80	271	270	42	42	257	300	3	29
C	120	275	284	46	56	270	308	16	37
D	160	298	299	69	71	288	305	34	34
E	200	312	—	83	—	271	301	17	30

is marked similarity between the two methods on serum A, the patterns given by the two methods differ in serum B. The change from the control cholesterol concentration, as measured by the micro method, is more consistent, although the figure to be taken as the cholesterol uptake (1) would be 34 and 37 mg./100 ml. for the macro and micro methods, respectively.

This example illustrates our experience that, although the micro method may give a pattern somewhat different from that given by the macro method, the micro method is perhaps the more consistent procedure. This characteristic is achieved together with the advantage that the micro method requires a total of only 1.4 ml. serum for determination of the cholesterol uptake, using a series of five incubated tubes plus two controls.

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