Effects of insulin on glucose metabolism in isolated human fat cells

R. B. GOLDRICK*

The Kanematsu Memorial Institute, Sydney Hospital, Macquarie Street, Sydney, Australia

ABSTRACT Isolated fat cells were used for the study of in vitro effects of insulin on glucose metabolism in human and rat adipose tissue.

In human subcutaneous fat cells, effects of insulin could be detected at concentrations of glucose in the medium from 1 to 10 μmoles/ml. Cellular responsiveness was inversely proportional to the glucose level. At a constant concentration of 6 μmoles of glucose per ml, the effects of insulin at various concentrations up to 500 μU/ml were investigated. At the highest concentration, which gave the maximal response, there was a 100% increase in the conversion of glucose-U-14C to glyceride-glycerol and a 40% increase in glucose oxidation. The dose-response curve was steepest between 2 and 20 μU/ml.

Rat epididymal fat cells were much more responsive to insulin. Glucose lipogenesis and pentose cycle activity could also be demonstrated in rat cells, whereas these activities could not be shown in fat cells from human omental and subcutaneous tissue. The findings for human cells are attributed to changes in cellular activity during preparation.

KEY WORDS glucose metabolism · isolated fat cells · human · rat · insulin

The rat epididymal fat pad has proved to be an extremely useful tool for studying the effects of insulin on lipid and carbohydrate metabolism in adipose tissue. It exhibits high rates of glucose lipogenesis in the basal state (1) and is sufficiently sensitive and reproducible in its response to insulin to serve as a bio assay for this hormone (2, 3). By comparison, human adipose tissue performs poorly in vitro (4–9); and only recently have changes in carbohydrate metabolism been observed at insulin levels equivalent to those of plasma immunoreactive hormone in vivo (10, 11). Furthermore, a convincing in vitro response to changes in insulin concentration over the physiological range has yet to be demonstrated in human adipose tissue.

The present investigation was undertaken to further explore the effects of insulin on glucose metabolism in adipose tissue from adult man. Isolated fat cells were employed in preference to intact tissues because epididymal adipose cells have proved to be more sensitive and reproducible in their responses to hormones than the fat pad (12). The results obtained here indicate that the uptake of glucose and its conversion to glyceride-glycerol by human fat cells are stimulated by concentrations of insulin similar to those assayed in plasma after glucose ingestion. However, glucose lipogenesis was notably absent in this preparation.

MATERIALS AND METHODS

Subjects

Fat cell suspensions were prepared from adipose tissue obtained from 9 males and 15 females between the ages of 20 and 79 yr (average age, 49 yr) who were undergoing surgery for gall bladder disease, peptic ulcer, appendicitis, or abdominal hernia. None showed evidence of diabetes mellitus, thyroid dysfunction, recent weight change, malnutrition, or gross obesity.

Rats

Male Sprague-Dawley rats (195–250 g) used in these studies were fed ad libitum on a pellet diet (Drug Houses of Australia, Rural Division, Sydney, Australia) consisting of 20% protein, 9% fat, and 50% carbohydrate, plus required vitamins and minerals.

Isotopes and Reagents

Labeled glucose (D-glucose-U-14C, D-glucose-1-14C, D-glucose-6-14C), crystalline insulins (human, lot 560-762B,
23 U/mg; porcine, lot PJ 5589, 23.9 U/mg), and various lots of bovine albumin (Fraction V) and collagenase were obtained commercially (glucose, Radiochemical Centre, Amersham, England; insulins, Eli Lilly & Co., Indianapolis, Ind.; albumin, Armour and Company Ltd., Eastbourne, England; collagenase, Worthington Biochemical Corporation, Freehold, N. J.).

Albumin–bicarbonate buffer was prepared as follows. A solution of bovine albumin, 0.1 g/ml water, was adjusted to pH 7.4 and dialyzed for 24 hr at 4°C under 95% lots of bovine albumin (Fraction V) and collagenase were chemical Corporation, Freehold, N. J.).

23 U/mg; porcine, lot PJ 5589, 23.9 U/mg), and various obtained commercially (glucose, Radiochemical Centre, Amersham, England; insulins, Eli Lilly & Co., Indianapolis, Ind.; albumin, Armour and Company Ltd., Eastbourne, England; collagenase, Worthington Biochemical Corporation, Freehold, N. J.).

Albumin–bicarbonate buffer was prepared as follows. A solution of bovine albumin, 0.1 g/ml water, was adjusted to pH 7.4 and dialyzed for 24 hr at 4°C under 95% O2–5% CO2 against two changes of Krebs-Henseleit bicarbonate buffer containing half the suggested concentration of calcium ion. Immediately before use the solution was diluted with an appropriate volume of bicarbonate buffer according to its measured protein content (13). Unless otherwise specified the final protein concentration was 0.04 g/ml and glucose was added to provide a concentration of 6 μmoles/ml. The albumin–bicarbonate buffer was shown to contain no measurable insulin by immunoassay (14). The addition of albumin (0.015 and 0.04 g/ml) to Krebs-Henseleit bicarbonate buffer also failed to accelerate the conversion of glucose-U-14C to glycerides and CO2 by human subcutaneous fat cells.

Removal of Adipose Tissue
Fat from the subcutaneous tissues of the anterior abdominal wall and the greater omentum was removed under general anesthesia. Morphine and atropine or their derivatives were employed for the premedication of subjects undergoing surgery. Anesthetic agents were used in various dosages and combinations, and included thiopentone sodium, nitrous oxide, morphine derivatives, succinyl choline, and d-tubocurarine. Some subjects were allowed to fast for up to 12 hr prior to surgery whereas others, who are specified in the text, received 1.5 liters of 5% glucose intravenously during this period. Tissues were obtained as soon as possible after induction of general anesthesia except when both subcutaneous and omental fat were studied in the same subject. Under these circumstances, removal of the subcutaneous fat was delayed to coincide with that of the omental specimen. Rats were sacrificed with carbon monoxide and the distal portions of the epididymal fat depots were removed. All tissues were placed in warm (37°C), gassed bicarbonate buffer and processed within 15 min.

Preparation of Isolated Fat Cells
Fat cell suspensions were prepared by the method of Rodbell (12) with the incorporation of the following modifications. (a) Siliconized glass incubation flasks and centrifuge tubes were used throughout. The silicone was applied as a 5% (w/w) solution of Dow Corning 200 fluid in chloroform and baked on at 300°C for 3 hr. (b) Tissues from all sources were incubated for 90 min at 37°C in albumin–bicarbonate buffer containing 10 mg of collagenase/ml in an atmosphere of 95% O2–5% CO2. Disappearance of the fat cells was facilitated by brief (2 sec) agitation on a vibratory mixer at 15-min intervals during the course of the incubation. After aspiration of free floating fat and residual adipose tissue, the cells were divided into 1-ml lots and thrice washed in 12-ml volumes of warm (37°C) albumin–bicarbonate buffer. Human fat cells were more fragile than those of the rat and readily broke down to lipid droplets during the washing procedure. This was preventable by careful handling at 37°C and by the use of freshly siliconized glassware.

Incubation Conditions
The cells were dispensed in 0.5 ml aliquots into siliconized 25-ml Erlenmeyer flasks equipped with center wells containing 0.5 ml of albumin–bicarbonate buffer. The latter contained both labeled and unlabeled glucose and appropriate concentrations of insulin. The flasks were gassed with 95% O2–5% CO2, capped with rubber serum stoppers, and incubated in a shaker water bath at 37°C for 2 hr. At least four replicate flasks were incubated in each experiment. Preliminary studies utilizing both human and rat fat cells showed that the incorporation of glucose-U-14C into glycerides and CO2 proceeded in a linear fashion for 2 hr.

Analytical Procedures
Radioactivity was measured in a Packard Series 3000 model liquid scintillation counter. 14C-radioactivity in lipids and CO2 was counted in a scintillation solution consisting of 0.4% 2,5-diphenyloxazole and 0.05% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene in toluene. Quenching was corrected for by the channels ratio technique. At the end of the incubation period 0.5 ml of Hyamine [p-(disobutylcresoxy-ethoxyethyl) dimethyl benzylamino] was injected into the center well and 0.2 ml of 11 N sulfuric acid into the cell suspension. 14CO2 was equilibrated according to the technique of Snyder and Godfrey (15).

For the determination of 14C-glycerides the cell suspensions were extracted with Dole’s “M” solution (16) and appropriate volumes of heptane and water were added. The lower phase was aspirated and the upper phase washed with preequilibrated, lower phase “M” solution. Aliquots of the upper phase were analyzed for carboxyl ester content (17), with tripalmitin as the standard, and for total lipid radioactivity. The latter has been shown previously to be confined to tri- and diglycerides in extracts of human adipose tissue (8). 14C in glyceride fatty acids was measured in an aliquot of the total lipid extract. The latter was saponified and the fatty acids were extracted into hexane after acidification as described by
Conversion of glucose to glycerides by subcutaneous fat cells increased 2-fold as the female aged were the same ing 1,

Under basal conditions the conversion of glucose to glycerides, glyceride-glycerol, and glyceride fatty acids were hexane phase after acidification exceeded 99.5\%.

Carrier demonstrated that the recovery of fatty acid in the total lipid extract was assumed to represent radioactivity in glyceride-glycerol. Studies with palmitic acid-1\(^{14}\)C plus unlabeled palmitic acid as carrier demonstrated that the recovery of fatty acid in the hexane phase after acidification exceeded 99.5\%.

The quantities of glucose converted to CO\(_2\), total glycerides, glyceride-glycerol, and glyceride fatty acids were calculated from the initial specific activity of the glucose in the medium and the quantity of radioactivity in the products. Results are expressed as \(\mu\)moles of glucose per mmole of triglyceride in the cell suspension.

### Statistical Analysis

Comparisons were made by the analysis of variance. When significant interactions occurred between such primary sources of variation as subjects and insulin the analysis was broken down into smaller components (18) or the mean square of the appropriate interaction was employed as the divisor in computing the variance ratio (19). Coefficients of variation of the differences between replicate flasks were computed from the residual sums of squares of several analyses of variance. These averaged 4% for the conversion of glucose-U-\(^{14}\)C to glycerides and 6% for its conversion to \(^{14}\)CO\(_2\).

## RESULTS

### Glucose Concentration of the Incubation Medium and the Rate of Glucose Uptake by Human Fat Cells

Under basal conditions the conversion of glucose to glycerides by subcutaneous fat cells increased 2-fold as the glucose concentration was increased from 1 up to 10 \(\mu\)moles/ml (Table 1). Under the same conditions, glucose oxidation was accelerated 6-fold. In the presence of insulin, there was a significant augmentation of glucose incorporation into glycerides at all tested levels of glucose in the medium. However, the magnitude of the hormonal effect declined as the glucose content of the medium was raised. \(^{14}\)CO\(_2\) production was stimulated by insulin at glucose concentrations between 1 and 6 \(\mu\)moles/ml. The percentage changes in glucose oxidation were somewhat less than those observed with glyceride synthesis, and for the most part appeared to be independent of the glucose concentration; at 10 \(\mu\)moles of glucose per ml no insulin effect on \(^{14}\)CO\(_2\) production could be demonstrated. These findings indicate that glucose uptake by adipose cells was influenced by the extracellular concentration of glucose and by the presence of insulin. In the present investigation experiments were routinely performed at a glucose concentration of 6\(\mu\)moles/ml (108 mg/100 ml) in order to simulate conditions under which insulin becomes operative in vivo.

### Dose–Response Relationships for Insulin in Human and Rat Adipose Cells

#### Human.

In Fig. 1 are shown the effects of different concentrations of insulin on the conversion of glucose-U-\(^{14}\)C to glycerides and CO\(_2\) by human subcutaneous fat cells. Both human and porcine insulins were used and produced similar effects. Hence the results were pooled. They were also normalized by conversion to percentages of the control values for comparison with the dose–response curves of epididymal fat cells to insulin (see...
Fig. 1. Conversion of glucose-\(^{14}\)C to glycerides and CO\(_2\) by human fat cells in the presence of various concentrations of insulin. Each point represents the mean ± 2 SEM (vertical bars) of glucose conversion to glycerides □ and to CO\(_2\) △.

Subcutaneous adipose tissue was removed under general anesthesia from three males (34, 51, and 59 yr) and five females (20, 44, 47, 52, and 76 yrs). Four studies were performed with human and four with porcine insulin. Similar dose-response curves allowed both sets of data to be pooled. Cells were incubated in four replicate flasks for 2 hr at 37°C in albumin-bicarbonate buffer containing 6 μmoles of glucose per ml.

below). Glyceride synthesis in human adipose cells was stimulated by as little as 2 μU of insulin per ml (P<0.01). The response was nonlinear, rising steeply at hormone concentrations between 2 and 20 μU/ml and levelling off as the insulin concentration was further increased. The mean value resulting from the highest level of insulin was 200% of the control. Glucose-\(^{14}\)C conversion to CO\(_2\) underwent a similar nonlinear acceleration as the insulin level was raised. Changes in CO\(_2\) formation were demonstrable in the presence of 10 μU/ml (P<0.01), but the mean increase for high doses was only 40% of control values. Changes in glyceride synthesis and glucose oxidation in response to insulin varied widely from one subject to another. The source of this variation was not explored in detail, but there was no obvious relationship between insulin response and the age or sex of the subjects. Hydrolysis of the glycerides in cells from two of the subjects. (male, aged 51 and female, aged 20 yr) revealed that changes in glyceride synthesis were solely attributable to accelerated conversion of glucose to glyceride-glycerol.

Rat. Fig. 2 illustrates the effects of various concentrations of insulin on fat cells pooled from several rats. Here, variations in response (standard deviations) represent the methodological error, as opposed to Fig. 1, where variations about the means (standard errors) represent both the population variance and the methodological error. The rat preparation responded to increasing hormone concentrations with a nonlinear enhancement of glucose-\(^{14}\)C conversion to CO\(_2\), glyceride-glycerol, and glyceride fatty acids. Here also the slopes of the curves were steepest at insulin concentrations between 2 and 20 μU/ml. Maximal values were reached at 40–80 μU/ml; and the minimal effective insulin concentration appeared to be 10 μU/ml. In contrast to human fat cells, rat adipose cells achieved a 15-fold increase in the synthesis of glyceride fatty acids and a 5- to 6-fold increase in glucose conversion to CO\(_2\) in the presence of insulin. On the other hand, the changes in glyceride-glycerol synthesis were similar to those observed with human fat cells. A large fraction of the insulin-induced conversion of glucose to CO\(_2\) in rat adipose tissue reflects enhanced function of the pentose cycle (1). The latter facilitates fatty acid synthesis (20), and its absence or inactivity in human fat cells would account for most of the observed differences in insulin response.

Glucose-\(^{14}\)C Metabolism in Rat Epididymal and in Human Omental and Subcutaneous Fat Cells

In order to obtain fair estimates of relative metabolic activity, we incubated omental and subcutaneous fat cells from each subject in parallel (Table 2). On every occasion, incubations of rat epididymal fat cells were also undertaken, primarily to assess the effects on the data of
possible day-to-day variations in experimental conditions. Glyceride synthesis and glucose oxidation were more rapid in omental than subcutaneous fat cells (Table 2; P<0.05). However, the differences were small: they were demonstrable only when the control and insulin data were pooled. Insulin stimulated the conversion of glucose to glycerides to an equal extent (about 2-fold) in both preparations. Its effects on glucose oxidation were of a low order of magnitude and attained statistical significance only in the case of omental fat cells.

It is interesting to note that the basal rates of glucose conversion to glycerides and CO₂ by rat epididymal fat cells were about seven and five times as great as those observed in both the human preparations. In the presence of insulin, these differences were magnified and reflected a 2- to 3-fold greater hormonal response in rat adipose cells.

The coefficients of variation listed in Table 2 show that intersubject differences in basal rates of glyceride and CO₂ production were 5–10% higher than in epididymal fat cells. In the presence of insulin the coefficients of variation increased in both species; but variations between subjects were then 18–27% higher than between rats. Thus, insulin responsiveness was lower and spontaneous variations in metabolic activity were higher in human than in rat adipose cells.

Scatter diagrams of the raw data from which Table 2 was constructed showed a close correspondence between the magnitude of the insulin effect on glyceride and CO₂ production in omental and subcutaneous fat cells from individual subjects. However, there was no indication of any relationship between the insulin effect on rat and human adipose cells incubated at the same time. This indicates that variations in insulin
from one rat. The cells were incubated for 2 hr in albumin-
subject were incubated in parallel with the epididymal fat cells

\[ \text{Conversion of glucose to CO}_2 \]

The data are consistent with an absence of depression of the pentose cycle in these preparations of human adipose cells. They may also account for the inconsistent demonstration of significant hormonal effects on the conversion of glucose-U-\textsuperscript{14}C to CO\textsubscript{2} in other experiments reported here.

**DISCUSSION**

The present investigation has shown that the conversion of glucose to glycerides and CO\textsubscript{2} by human adipose tissue is facilitated by insulin in vitro. While these observations are by no means novel, the use of isolated fat cells as opposed to whole tissues has demonstrated hormonal effects at lower levels than previously reported. Owen, Lindsay, Gaskin, and Hollifield (10) recorded increased glucose oxidation by human subcutaneous fat in the presence of 31 \mu U of insulin per ml; and Björntorp (11) has obtained enhanced glyceride-glycerol formation with 10 \mu U/ml. In other investigations pharmacological concentrations of hormone have been employed in vitro (5, 21–25) to effect changes in carbohydrate metabolism. In this study human fat cells were sensitive to as little as 2 \mu U of insulin per ml. Near maximal effects were observed with 40 \mu U/ml, which lies in the range of plasma insulin levels recorded in man after the ingestion of glucose (26). The response to increasing concentrations of hormone was sigmoid. Similarly shaped dose–response curves were demonstrated for the effect of insulin on rat epididymal fat cells; the results substantiate the observations of others using isolated fat cells (27) or whole tissues (28) from the rat.

In previous reports on the effects of insulin on the metabolism of human adipose tissue the concentrations of glucose used in the incubation media have varied over a wide range (8–11, 21–25). Whereas high levels of glucose do not appear to depress the insulin-induced
acceleration of glucose uptake by adipose tissue (29) or isolated fat cells (12) from the rat, the situation appears to be somewhat different in man. Björntorp and Martinsson (30) were able to demonstrate an insulin effect on glucose conversion to glyceraldehydes by subcutaneous adipose tissue only at levels of glucose in the medium below 5 μmoles/ml. Similar findings were reported by Fessler, Beck, and Rubinstein (22) using omental fat. This investigation has shown that the conversion of glucose to glyceraldehyde by subcutaneous fat cells is facilitated by insulin at medium glucose concentrations between 1 and 10 μmoles/ml. Even so, the magnitude of the response declined with increasing levels of substrate because of a concomitant and disproportionate rise in the basal rates of glucose uptake. In the case of glucose oxidation, the basal and insulin-stimulated rates increased in parallel as the glucose level of the incubation medium was raised. Nevertheless, at 10 μmoles of glucose per ml, insulin failed to augment glucose oxidation.

The virtual absence of pentose cycle activity and of fatty acid synthesis from glucose in human omental and subcutaneous fat cells clearly distinguishes this in vitro preparation from the corresponding preparation of human adipose tissue. With the latter, glucose lipogenesis is almost always demonstrable in the isocaloric state (8, 11, 21); it responds to changes in caloric balance (31) and is impressive in patients with Type IV hyperlipoproteinemia (32). The present negative findings with glucose lipogenesis were neither related to the nutritional status of the subjects nor influenced by insulin in vivo. In contrast, rat epididymal adipose cells prepared and incubated under conditions identical with those for human cells exhibited higher over-all rates of glucose metabolism, converted glucose to glyceride fatty acids in the basal state, and exhibited the well-documented in vitro responses to insulin. They also appeared to be more robust than human fat cells, which tended to rupture easily unless carefully handled during preparation. In several respects, the observed behavior of human fat cells was similar to that of epididymal adipose cells traumatized by sonication or shaking with silicic acid (33). It is therefore tentatively suggested that the human fat cells prepared here were not analogous to intact epididymal fat cells, but exhibited some of the characteristics of an unfortified homogenate.

Comparative studies on omental and subcutaneous fat cells were prompted by earlier reports that the metabolic activity of human omental adipose tissue is greater than that of subcutaneous fat (7, 9, 21). Since the latter is the more fibrous and loculate of the two, it was reasoned that differences in accessibility of the cell mass to the

<table>
<thead>
<tr>
<th>Source of Cell Suspension</th>
<th>Presence of Insulin</th>
<th>Conversion of Glucose-1-14C to Glyceride-Glycerol</th>
<th>Conversion of Glucose-1-14C to Glyceride-Glycerol</th>
<th>Conversion of Glucose-6-14C to Glyceride-Glycerol</th>
<th>Conversion of Glucose-6-14C to Glyceride-Glycerol</th>
<th>C-1: C-6 in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Epididymal fat</td>
<td>None</td>
<td>Human</td>
<td>0.67 ± 0.028</td>
<td>1.64 ± 0.536</td>
<td>3.92 ± 0.505</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>4.49 ± 1.383</td>
<td>21.09 ± 1.207</td>
<td>31.14 ± 0.613</td>
<td>7.19 ± 0.911</td>
<td>42.21 ± 2.368</td>
</tr>
<tr>
<td>Subject A</td>
<td>Subcutaneous fat</td>
<td>None</td>
<td>Human</td>
<td>0.19 ± 0.022</td>
<td>0</td>
<td>0.24 ± 0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.24 ± 0.028</td>
<td></td>
<td>0</td>
<td>0.29 ± 0.036</td>
<td>0.27 ± 0.015</td>
</tr>
<tr>
<td>Subject B</td>
<td>Subcutaneous fat</td>
<td>None</td>
<td>Human</td>
<td>0.25 ± 0.005</td>
<td>0</td>
<td>0.25 ± 0.017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 ± 0.017</td>
<td></td>
<td>0</td>
<td>0.34 ± 0.014</td>
<td>0.50 ± 0.022</td>
</tr>
<tr>
<td>Omental fat</td>
<td>None</td>
<td>0.57 ± 0.079</td>
<td></td>
<td>0.37 ± 0.066</td>
<td>0.32 ± 0.016</td>
<td>0.08 ± 0.006</td>
</tr>
<tr>
<td>Subject C</td>
<td>Subcutaneous fat</td>
<td>None</td>
<td>Porcine</td>
<td>0.36 ± 0.009</td>
<td>0</td>
<td>0.50 ± 0.034</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.46 ± 0.004</td>
<td></td>
<td>0.82 ± 0.064</td>
<td>0.44 ± 0.039</td>
<td>0.13 ± 0.049</td>
</tr>
<tr>
<td>Omental fat</td>
<td>None</td>
<td>0.34 ± 0.017</td>
<td></td>
<td>0.48 ± 0.023</td>
<td>0.37 ± 0.020</td>
<td>0.10 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>Porcine</td>
<td>0.65 ± 0.038</td>
<td></td>
<td>0.77 ± 0.009</td>
<td>0.63 ± 0.006</td>
<td>0.12 ± 0.013</td>
</tr>
</tbody>
</table>

* Incubation was for 2 hr in albumin-bicarbonate buffer containing 6 μmoles of glucose per ml; insulin added, 100 μU/ml.
† Cell suspensions were prepared from three rats (182, 195, and 212 g) and incubated in parallel with cells from Subject A (female, 73 yr). Suspensions from Subjects B and C (male, 52 yr, and female, 73 yr) were incubated on separate days. All subjects received 1.5 liters of 5% glucose during 12 hr prior to surgery.
‡ Fatty acid synthesis was recorded as zero when glucose-14C conversion was less than 0.01 μmoles/mmol triglyceride in 2 hr.
§ Mean values from four replicate flasks ± se.
incubation medium might account for higher reported rates of metabolic activity in the omentum. These assumptions were borne out to some extent in that glucose metabolism in omental fat cells exceeded that in subcutaneous fat cells by only a small margin. Since subcutaneous and omental adipose cells may differ in size (34), it is possible that the present findings were due to differences in cellular lipid. However, definite conclusions on this point would be justified only from metabolic data expressed in terms of the DNA or protein content of isolated fat cells.

The author thanks Miss Jennifer Ellen for her capable technical assistance and Dr. Bernard Lake of Lilly Industries Pty. Ltd. for the generous supplies of human and porcine insulin.

This study was supported in part by Grants-in-Aid G394 and G395 from the National Heart Foundation of Australia. Manuscript received 29 May 1967; accepted 7 August 1967.

REFERENCES