Increased Lipid Synthesis by Liver Slice in a Superfusion System Following Raised Glucose or Insulin Concentration

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Summary. The synthesis of lipid from ^{14}C-glucose and ^{3}H-alanine was measured from rat liver slices using a micro-superfusion technique that allows continuous monitoring of the perifusate output. Increase in substrate glucose results in prompt elevation of fatty acid synthesis. Insulin addition also produced an increased ^{14}C incorporation into lipid. The effects were independent. Both these factors may be active in the hypertriglyceridaemia of high carbohydrate intake or diabetes.

Augmentation de la synthèse des lipides par des coupes de foie dans un système de superfusion après élévation de la concentration de glucose ou d'insuline

Résumé. La synthèse des lipides à partir du ^{14}C-glucose et de la ^{3}H-alanine fut mesurée dans des tranches de foie de rat, en utilisant une technique de microsuperfusion qui permettait l'enregistrement continu du débit du périfusat. L'addition de glucose amena une hausse immédiate de la synthèse d'acide gras. L'addition d'insuline provoqua de même une augmentation de l'incorporation de ^{14}C dans les lipides. Les effets étaient indépendants. Ces deux facteurs pourraient être actifs dans l'hypertriglycéridémie après l'absorption importante d'hydrates de carbone ou dans l'état diabétique.

Erhöhte Lipidsynthese von Leberschnitten in einem Superfusionssystem nach erhöhten Konzentrationen von Glucose und Insulin

Zusammenfassung. An Rattenleberschnitten wurde mit einer Mikrosuperfusionstechnik, die eine dauernde Kontrolle des Perfusatflusses erlaubt, die Lipidsynthese aus ^{14}C-Glucose und ^{3}H-Alanin gemessen. Eine Erhöhung des Glucosesubstrats führte zu einer sofortigen Steigerung der Fettsäuresynthese. Zugegebenes Insulin erzeugte ebenfalls einen erhöhten ^{14}C-Einbau in die Lipide. Beide Effekte waren unabhängig. — Diese beiden Faktoren können in der Hypertriglycerinämie nach hoher Kohlenhydrataufnahme oder beim Diabetes wirksam sein.

Introduction

Recent advances in the study of lipid metabolism have revealed an intimate interrelationship with the metabolic pathways of carbohydrate utilisation. The hyperlipidaemia associated with some cases of uncontrolled diabetes is well known, as is the pathological state of carbohydrate induced lipaemia in man [1, 2]. In normal volunteers the administration of an isocaloric diet containing > 7 g/kg carbohydrate results in initial elevation of circulating triglycerides, and reduced clearance of an exogenous triglyceride load [3]. Some experiments suggest that the type of carbohydrate fed may further influence the degree of hyperlipaemia [4].

The circulating triglyceride at any given time is derived from two sources: the intestine and the liver. In the post-absorptive state the principle source of circulating triglyceride is the liver, where esterification of both circulating and de novo synthesised fatty acids is succeeded by coupling to a protein and excretion into the plasma, chiefly as very low-density lipoproteins (VLDL).

High circulating free fatty acid levels have been shown to be associated with increasing hepatic output of triglyceride [5], and this mechanism may contribute to the hypertriglyceridaemia associated with Cushing's disease [6]. The administration of a high carbohydrate diet would, however, be expected to reduce circulating free fatty acid levels, implying other mechanisms for the hyperlipaemia observed in this condition. These may include direct stimulation by the absorbed carbohydrate of hepatic lipid output, or its secondary increase by higher circulating insulin levels.

In the choice of an experimental system for the in vitro study of hepatic lipid synthesis, established experimental models have their limitations. Tissue slice experiments of classical design do not allow for the sequential study of the effect of hormone and substrate changes, while perfused whole liver experiments are by definition unsuitable for use with biopsy specimens.

We have investigated further [6a] the applicability of a continuously superfused rat liver slice system as an experimental model for the study of some factors relevant to diabetic and carbohydrate induced hyperlipaemia.

Materials and Methods

Krebs ringer bicarbonate was prepared according to the standard technique [7] but with K^+ and Na^+ concentrations set at 70 and 53 mEq/litre respectively [8]. Two percent w/v recrystallised bovine serum albumin (Armour) was added and the pH checked to

7.4. Uniformly labelled ^{14}C-glucose or ^{3}H-alanine (Radiochemical Centre, Amersham, Buckinghamshire) was added with unlabelled substrate to final concentrations of 1.25 to 80 mM (1 μCi ^{14}C/ml) glucose and alanine 0.80 mM (1.33 μCi/ml). Blood bank human plasma was separated from erythrocytes by gentle centrifugation at 4° and diluted 1 : 1 with buffer to which glucose was added to a final concentration of 10 mM.

Male wistar rats of 150—200 g wt. fed ad lib on a standard laboratory diet, were stunned by a blow on the head, decapitated, and the livers immediately placed in ice cold 0.9% saline solution. Liver slices were prepared (0.4 mm thickness, approx. 200 mg) with a Stadie-Riggs microtome and washed in saline solution at 4° before addition to the superfusion chamber [9]. The slow perfusion peristaltic pump delivered approximately 3 ml/15 min through the incubation chamber, from a 80 ml reservoir maintained at 37°C and the superfusate was fraction-collected in graduated centrifuge tubes without re-cycling after an initial 20 min equilibration. Where relevant perfusate reservoirs were interchanged at mid-point in the experiment, and insulin addition made either by addition to the reservoir or by injection into a sidearm on the afferent circuit.

After collection the superfusate was extracted into five volumes Dole medium [10]. The heptane phase
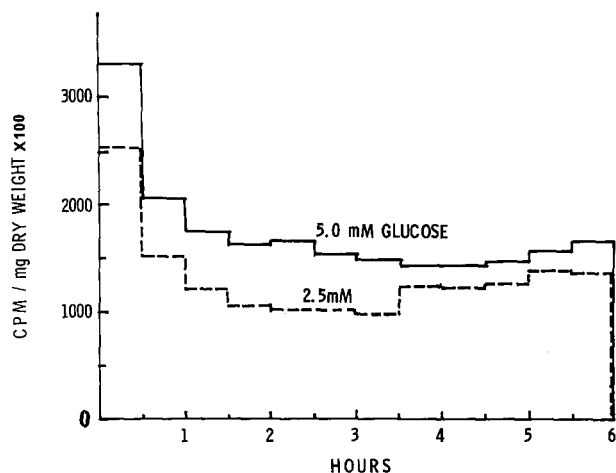
for streaking and thin lay chromatography using hexane : diethyl ether : acetic acid : methanol (80 : 20 : 2 : 3) on Eastman chromatogram sheets, 20 cm X 20 cm.

At the end of superfusion the liver slices were blotted, weighed and extracted in chloroform-methanol (2 : 1) [11]. After addition of 0.4 volumes of 0.05 M NaCl and separation of the phases, the lower chloroform phase was washed three times and aliquots dried down for chromatographic fractionation as for the superfusate.

In the classical liver slice experiments, slices of liver (500 mg) were incubated in identical experimental buffer (3 ml) for 2 h in a shaker (30 cycles/min) at 37° in an atmosphere of 95% O_2, 5% CO_2. The slices were blotted, weighed and the medium and slices processed respectively as described above. Boiled liver slices did not show significant activity, and re-extraction of Folch-extracted liver slices yielded insignificant radio-activity.

Results

Superfused liver slices incorporate ^{14}C-labelled glucose into lipid at a relatively constant rate after the first 30 min (Fig. 1), and this is maintained for up to 6 h in the absence of stimulating factors. Replacement
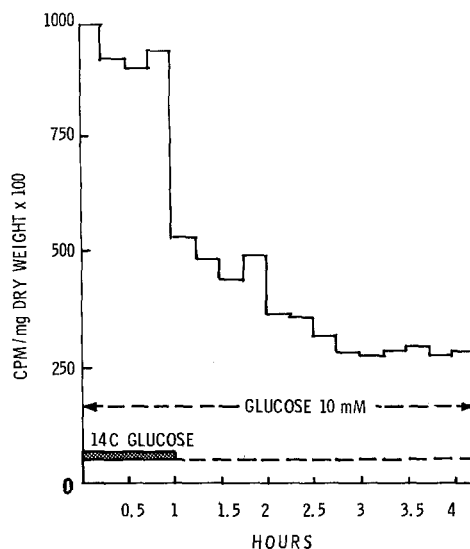

Fig. 1. *Perifusion:* Baseline incorporation of ^{14}C-glucose into lipid. Liver slices were prepared as described in Methods, and incubated in superfusion chambers in modified Krebs-Ringer buffer medium with glucose 2.5 mM --- and 5 mM — — — (1 μC/ml), (Means of two experiments)

Fig. 2. *Perifusion:* ^{14}C-labelling of perifusate lipid following equimolar substitution of ^{14}C-glucose by non-radio-active glucose both at 10 mM after the first hour. (Mean of two experiments)

was washed three times with 0.4 volumes 0.03 N. sulphuric acid, aliquots dried under a steam of hot air and counted in toluene PPO-POPOP (containing 4 g 2.5 diphenoxazole and 0.4 g p Bis-(2-(5-phenyloxazolyl) benezene per litre of AR grade toluene) in a Packard scintillation counter (Model 3320). For separation into lipid fractions further aliquots were dried down under nitrogen and resuspended in 20 μl chloroform

of ^{14}C-labelled substrate with equi-molar unlabelled glucose is followed by a rapid fall in lipid labelling (Fig. 2). Superfusion of the two adjacent incubation chambers containing liver slices from the same rat with buffers of differing glucose levels result in incorporation of ^{14}C-labelled glucose into lipids in approximate proportion to the differences in substrate glucose concentration (Fig. 3). Exchange of buffer

reservoirs between the two chambers after 2 h, allowing 15 min for re-equilibration, resulted in rapid change in output of ^{14}C-glucose-labelling between the two chambers, and in lipid output to a new steady-state of similar proportion to the differences in substrate concentration. In a later group of experiments, doubling
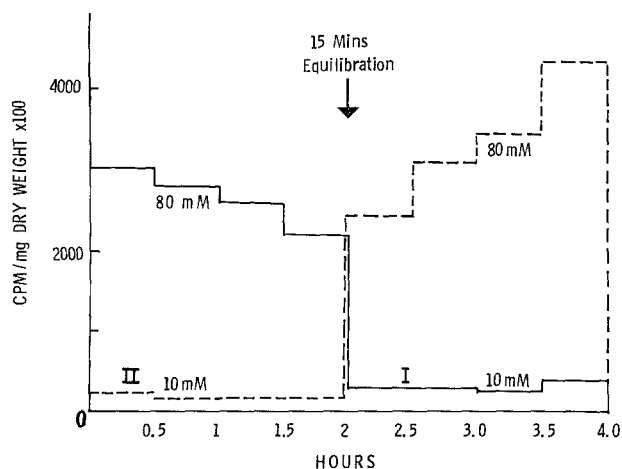

Fig. 3. *Perifusion:* Glucose ^{14}C incorporation into superfusate lipid following reservoir exchange between buffers of glucose levels: 10 mM and 80 mM. (Two of a number of similar experiments). Perifusion chambers I — — — and II - - -

Further modification of the superfusate by the addition of human plasma (1 : 1 as defined in "Methods") produced a 12-fold increase in ^{14}C-glucose incorporation into lipid (Fig. 6) and cross-superfusion after 3 h resulted in stimulation of the slices in the chamber receiving the plasma enriched superfusate, while 3 h superfusion with standard Krebs buffer did not result in return of the plasma-poor slices to the lower level of lipid synthesis.

In contrast to the relatively brisk arrival at new steady-state in lipid synthesis by liver slices superfused with medium of varying carbohydrate or plasma enrichment, the response to the addition of insulin is slower, and progressive over the 4 h following hormone injection (Fig. 7). Differing levels of glucose in the medium between the chambers containing slices from the same rat were reflected in proportionally differing basal values, but the difference is less apparent in the superfusate fractions following insulin addition. Insulin addition furthermore increased ^{3}H-alanine incorporation into lipid under similar experimental conditions (Fig. 7b).

Thin layer fractionation of lipid extracts of tissue and superfusate reveal, however, a difference between the system under study and the more classical liver slice experiments. Whereas ^{14}C-glucose carbon is incorporated principally into triglyceride and phospho-
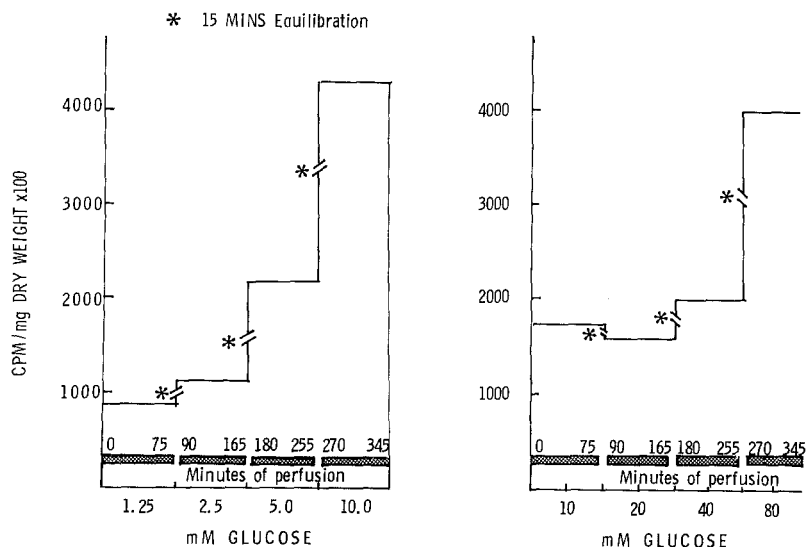

Fig. 4. *Perifusion:* The effect of sequential increase in superfusate glucose on ^{14}C-incorporation into superfusate lipid (mean of four experiments); glucose concentrations 1.25, 2.5, 5.0 and 10.0 mM, and in a second series of experiments 10, 20, 40 and 80 mM

of substrate concentration was undertaken in pairs of chambers, allowing 15 min for equilibration between 75 min sampling periods (Fig. 4). Concentrations investigated ranged from 1.25 to 80 mM and over this range the ^{14}C-glucose incorporated by liver slices was increased with the increasing substrate glucose concentrations. These changes have been confirmed in the classical liver slice experiments (Fig. 5).

lipid fractions of tissue extracts in both superfusion and classical type experiments, isotopic incorporation of superfusate extracts is principally into the free fatty acid fraction, with differences only resulting between the addition of glucose, insulin or half-strength plasma (Table 1). In similar designed experiments to those described, (Tulloch — unpublished data) the triglyceride/free fatty acid ratios were not altered by further

addition of aminoacid mixture matching rat plasma composition, while palmitate addition above 1.0 mM decreased the total ^{14}C-glucose carbon incorporation into superfusate lipids. Other hormones, including ACTH (200 mU/ml), growth hormone (1 μg/ml),

Discussion

The superfusion system allows sequential sampling of the lipid output from small quantities of hepatic tissue such as would be obtained by operative biopsy
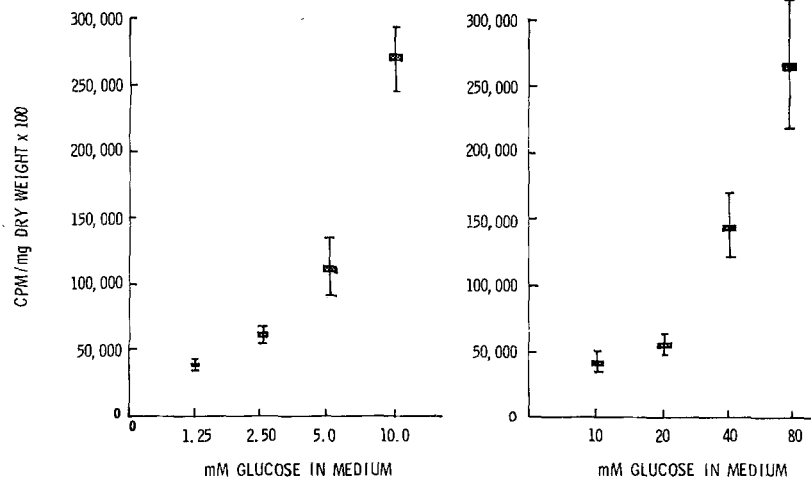
Fig. 5. *Liver slices:* The effect of increasing glucose concentration on substrate incorporation into lipid (each point mean of 20 experiments). Glucose concentrations 1.25, 2.5, 5.0 and 10.0 mM, and in a second series of experiments 10, 20, 40 and 80 mM
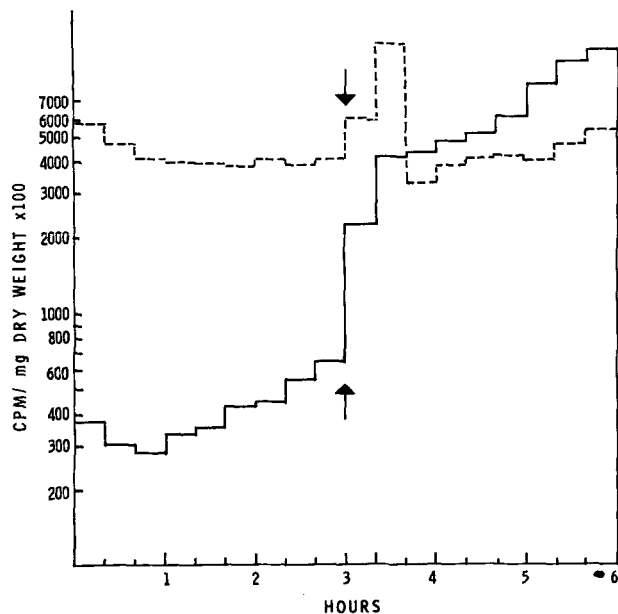
Fig. 6. *Perifusion:* The effect of half-strength human plasma on glucose incorporation into superfusate lipid (two typical experiments of four). At points indicated by arrows, chambers were cross-perifused and an equilibration period allowed of 15 min. Glucose concentration 10 mM, 1.0 μC/ml

adrenaline (2—20 ng/ml), noradrenaline (10—100 ng/ml), hydrocortisone (20—200 μg/ml) and triiodothyronine (0.1—1.0 μg/ml) were without discernible effect when added to the in vitro system at the concentrations indicated.
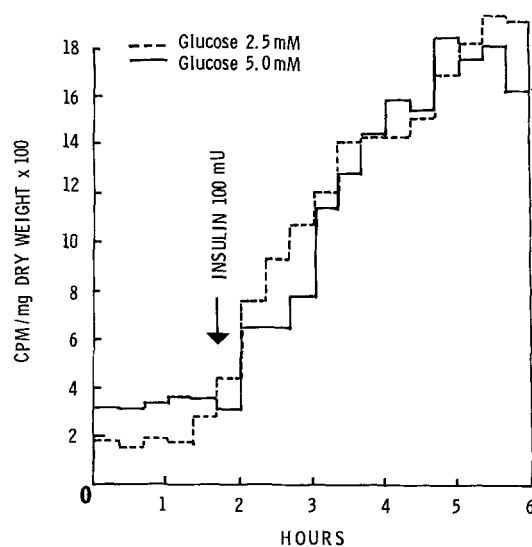
Fig. 7a. *Perifusion:* The effect of insulin addition on ^{14}C-glucose incorporation into superfusate lipid (two of six experiments). Glucose concentration 2.5 mM --- and 5.0 mM — — — in Krebs bicarbonate buffer

(200—600 mg wet weight). Under sterile conditions a stable baseline of ^{14}C-glucose incorporation into lipid persisted up to 6 h, and response has been demonstrated to changes in the superfusion medium by increased de novo synthesis by the liver of free fatty acids.

The importance of hepatic de novo synthesis of plasma free fatty acid varies between the species. In man and rabbit, the principle source of plasma triglyceride fatty acid is circulating plasma free fatty acid

[2, 5, 12]. In the rat, however, only a minor fraction of the free fatty acid originates from this pool, and quantitatively the most important source is hepatic lipogenesis [22]. In man under specific circumstances such as low fat, high carbohydrate diet, however, the
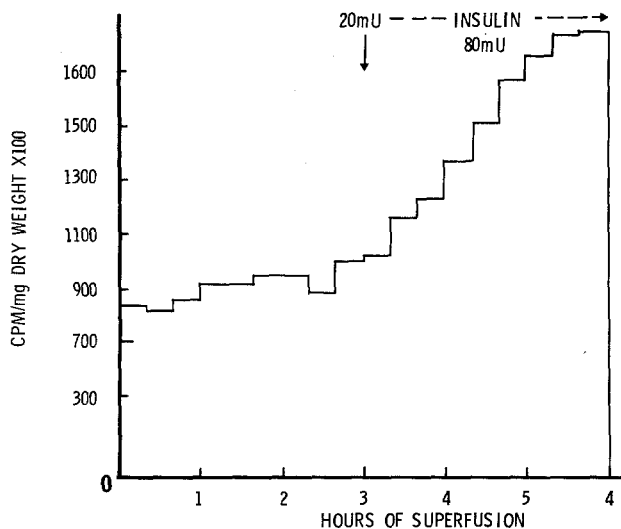

Fig. 7b. *Perifusion:* The effect of insulin addition on incorporation of ^{3}H-alanine into perifusate lipid (one of three experiments). Alanine 0.80 mM, 1.33 μCi/ml in modified Krebs bicarbonate buffer

the portal circulation. The role of insulin in increased *in vivo* triglyceride synthesis remains contentious and it is of interest that whereas the stimulation of both glucose and alanine into lipid could readily be demonstrated using the liver slice perifusion system, such stimulation was not demonstrable using the classical liver slice incubation. Liver insulinase would be more effective in the classical liver slice procedure, which may explain both this difference in our findings with the two procedures, and why this insulin effect has been observed only by some other workers using high insulin doses in vivo and in vitro [14].

The lipid synthesis response in rat liver to increased glucose concentration is prompt and similar to that described for increased glycogen synthesis [15]. High carbohydrate feeding of rats has resulted in increased hepatic levels of pyruvate, acetyl CoA and malate [16] while these authors also noted that ^{14}C conversion from fructose to fatty acids further exceeded that from glucose, suggesting that fatty acid synthesing enzymes are unlikely to be the rate-limiting factor in glucose conversion. In both glucose and fructose-fed groups, hepatic CoA carboxylase levels were elevated, as were gluco- and fructokinase activity. The brisk response in lipid synthesis in the experiments described here do not suggest de novo enzyme protein synthesis as a mechanism; but rather acceleration of an already active fatty acid synthesis, probably from

Table 1. *Thin layer fractionation of perifusate and tissue slice extracts*

Perifusate	No. of observations	% of counts occurring as:		
		Phospholipid	Triglyceride	FFA
Glucose 1.25 mM	12	6.2±0.61	7.2±0.81	86.9± 3.52
Glucose 2.5 mM	12	6.7±0.42	10.6±1.20	82.8± 6.30
Glucose 5.0 mM	12	9.9±0.88	10.0±3.21	80.1±10.92
Glucose 10.0 mM	12	8.2±1.23	13.1±0.21	75.1±15.38
Half-strength plasma				
1—3 h	9	23.7±2.88	5.9±0.21	70.4± 5.31
3—6 h	9	39.4±4.21	7.8±0.42	53.8± 4.29
Pre-insulin	6	16.4±3.61	8.3±1.51	72.5± 6.75
Insulin 1 h	6	28.3±4.92	6.8±0.09	64.8± 5.68
Insulin 2 h	6	35.9±2.61	6.2±0.94	58.0± 2.97
Insulin 3 h	6	22.3±1.75	6.4±0.37	71.0± 4.29
Tissue extracts of super- fusion expts.	12	18.7±0.92	73.5±6.61	7.8± 1.23
Liver slice lipids	13	15.4±0.32	80.3±4.37	4.3± 0.09

Slices and perifusate aliquots were extracted and concentrated as described in methods, and separated by thin layer chromatography as detailed using known standards.

importance of hepatic de novo free fatty acid synthesis is probably greater, since the principle increase in lipoproteins is the VLDL fraction and a similar situation may occur in cases of carbohydrate induced hyperlipaemia [13, 23]. Should this be the case, then the findings reported here may well indicate a mechanism for the high levels of circulating triglyceride secondary to increased glucose concentrations within

the increased glucose entering the hepatocyte, for it is permeable to glucose and has an active low-affinity glucokinase. Half-strength plasma addition to the perifusate similarly caused a brisk rise in FFA synthesis from glucose, and this persisted up to 3 h after cross-superfusion with Krebs buffer.

By contrast, the response obtained following insulin addition without change in glucose concentration,

was slower but progressive over the duration of the study. The site of insulin action is unknown, but it probably involves facilitating either glucose entry into cells, or its early intermediate metabolism. In the very permeable liver cell insulin effect may fall at one or more early points in glucose utilisation — e.g. phospho-fructokinase, reduced levels of which have been reported in human diabetic adipose tissue [17]. However these effects in the hepatocyte must not only provide more substrate but also accelerate fatty acid synthesis in other ways, for we have seen it induces increased incorporation of ^{3}H-alanine from the perifusate, and ^{14}C incorporation from aminoacids into fatty acid carbon is reduced in perfused livers from acute and chronic alloxan diabetic rats [24].

The significance of the above effects in the clinical situation of diabetic and nutritional hypertriglycer-idaemia is difficult to assess. Although insulin in high dose affects in vivo lipid synthesis in rats [14], and some workers have reported elevated glucose and insulin levels in idiopathic hypertriglyceridaemia [18], some normal levels [19], and some decreased levels have also been described [13, 20]. In the latter situation, decreased triglyceride clearance by peripheral tissues may be an important factor, since both adipose tissue lipoprotein lipase and intracellular alpha-gly-cerophosphate levels (for re-esterification) would provide insulin dependent factors for limiting trigly-ceride removal from the circulation.

The remaining differences between classical in vitro liver slice techniques and the superfusion system is the consistent incorporation of labelled precursor mainly into free fatty acid by the superfused slice. Since the major lipid output of the liver physiologi-cally is triglyceride linked with apoprotein in very low density lipoproteins, component precursors were added both in the form of aminoacid mixtures approximating to rat plasma components, and also half-strength human plasma (Fig. 6 & Table 1). Neither, however, was able to alter the free fatty acids to triglyceride ratio of the superfusate radioactivity although insulin and plasma increased the percentage of counts present as phospho-lipid, which could include phosphatidic acid. That an absence of cofactor or apoprotein accep-tor remains the most likely reason for this deficit is further substantiated by the studies of Kook & Ruben-stein [21] on increased rat liver slice output of phos-pholipid in media of various lipoprotein constituents.

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