STUDIES ON NUTRITION AND THE
ACUTE PHASE PLASMA PROTEIN RESPONSE.

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ABSTRACT

Malnutrition is associated with an increased incidence of morbidity and mortality after surgical operations. The acute phase plasma protein response is believed to be an important mechanism in the repair and resolution of inflammation following operation. This study attempted to determine if the acute phase response is attenuated by malnutrition and thus a possible contributor to the observed increase in the incidence of complications after surgery.

Four groups of patients were studied. Normally nourished patients with benign disease of the upper gastrointestinal tract, normally nourished patients with malignant disease of the oesophagus or stomach, malnourished patients with malignant disease of the oesophagus or stomach and malnourished patients with malignant disease of the oesophagus or stomach who had received intravenous nutrition for 7 days prior to operation.

Firstly, nutritional indices of the patients were assessed. Then, in the in vivo study, the acute phase proteins C-reactive protein, alpha\(_1\)-antitrypsin, and alpha\(_1\)-acid glycoprotein were measured sequentially following radical surgery for upper gastrointestinal carcinoma in groups 2, 3 and 4. Finally liver biopsies from all 4 groups were incubated in vitro in the presence of interleukin-1 an acute phase stimulant and the production of C-reactive protein measured using an ELISA.
assay.

The magnitude of the acute phase plasma protein response *in vivo* and *in vitro* was compared amongst the groups and correlations were sought between the magnitude of the acute phase plasma protein response *in vivo* and *in vitro* and the various nutritional indices.

No influence of nutritional factors could be demonstrated in either arm of the study.

This could be a genuine finding or the result of inadequacy in the design of the study or the power of the study in relation to the number of patients studied.

Further studies using methods to improve the preservation of the liver slices by shorter incubation and improved oxygenation are suggested.
DECLARATION

This thesis was composed entirely by myself. The initial idea to study the acute phase response was suggested by Mr M J McMahon, Senior Lecturer in the Department of Surgery, University of Leeds but the experimental design, the setting up of the organ culture, the liver processing, the data collection and the analysis of the results are all my own work carried out whilst employed as a Research Fellow in the University Department of Surgery at Leeds General Infirmary, between July 1986 and April 1988. The biochemical assays of CRP and other substances were performed for me by various departments in the University of Leeds. The manuscript was typed entirely by myself.

Elements of this work have been presented at the following meetings:

Surgical Metabolic Group, Manchester, November 1986
Leeds Regional Surgical Club, Scunthorpe, March 1987
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Las Vegas, February 1988
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LIST OF ABBREVIATIONS

AAG alpha\textsubscript{1}-acid glycoprotein
AAT alpha\textsubscript{1}-antitrypsin
C Celsius
C\textsubscript{1} the first component of complement
CRP C-reactive protein
D Dalton
DMEM Dulbecco's modified Eagle medium
DNA deoxyribonucleic acid
ELISA enzyme linked immunosorbent assay
EP endogenous pyrogen
g gram
HSF hepatocyte stimulating factor
IFN interferon
IgG immunoglobulin class G
IL-1 interleukin-1
l litre
LAF lymphocyte activating factor
LEM leukocyte endogenous mediator
m metre
PNI prognostic nutritional index
PRE prealbumin
rIL-1 recombinant DNA produced interleukin-1
SAA serum amyloid A
TNF tumour necrosis factor
TRF transferrin
INTRODUCTION
1. THE ACUTE PHASE PROTEINS

The acute phase response is a multi-system, non-specific response to almost any form of tissue injury including surgical trauma and bacterial infection, [Kushner, 1982]. It may be regarded as the systemic equivalent of the local inflammatory response, the purpose of which is thought to be to aid tissue repair, resist further tissue damage and resolve the inflammatory process. Its manifestations include fever, leukocytosis, changes in the hormonal milieu and blood coagulability, increased proteolysis in muscle, and alterations in gluconeogenesis and lipolysis [Dinarello and Wolf, 1982; Egdahl et al, 1977; Clowes et al, 1983; Wolfe, 1987; Wiener et al, 1987]. In addition there are rapid changes in the plasma concentrations of a number of circulating proteins, the so called acute phase proteins. These changes are often in themselves referred to as the acute phase response but more precisely should be called the acute phase plasma protein response.

Many of these proteins, e.g. fibrinogen, are normally present in the plasma and have a physiological role which is well understood. Others, of which C-reactive protein (CRP) is the prime example in man, appear in the plasma in significant concentration only during the acute phase response and their function remains incompletely understood. However, there is growing evidence to suggest that the acute phase proteins act as modifiers of the inflammatory response, acting as inflammatory mediators,
enzyme inhibitors, immune regulators and scavengers of effete macromolecules [Whicher and Dieppe, 1985].

The first step in the acute phase response is the attraction of mononuclear phagocyte cells to sites of inflammation. Here contact with micro-organisms, and dead tissue triggers the release of cytokines which act as the intermediate messengers for the many facets of the acute phase response. Thought initially to number only one, interleukin-1, there are now at least 6 cytokines known to influence acute phase protein synthesis.

A diagrammatic representation of the pathways of the acute phase response is given in figure 1, page 12.

The definition of what may be regarded as an acute phase protein is rather imprecise, since the concentration of many if not most plasma proteins changes in response to trauma and sepsis. Kushner proposed a definition based on a rise of at least 25% in plasma concentration, but conceded that a definition based on synthesis would be more satisfactory [Kushner, 1982], since increased synthesis may be matched by increased consumption thus masking the acute phase nature of proteins such as alpha2-macroglobulin, one of the few proteins which in man shows no change in plasma concentration during the acute phase response. Others have used the term more widely and even include proteins whose concentration falls as so called 'negative acute phase' proteins [Lebreton et al, 1979].

If we accept the condition of a rise of at least 25%, acute phase reactants can be broken into subgroups based
Figure 1. A simplified diagram of the pathway of initiation of the acute phase plasma protein response.
on the relative size of the change in concentration. Some proteins increase their concentration by several thousand fold, some by only a few fold, some by a factor less than one. The principle positive reactants are listed in table 1, page 14 and four of these proteins are discussed individually below. C-reactive protein is the best known and longest studied of the acute phase proteins in man and will be considered in most detail. Serum amyloid A shares some characteristics with CRP in that it is normally present in trace amounts but increases its concentration by a factor of thousands during acute phase stimulation. Alpha\(_1\)-antitrypsin and alpha\(_1\)-acid glycoprotein are also typical acute phase reactants and will be discussed on account of their role in the experimental section of this thesis.

C-REACTIVE PROTEIN

C-reactive protein is the archetypal acute phase protein in man. It is normally present in levels which until recently were undetectable but following injury or infection its concentration rises within a few hours to a level which may be several hundred times higher than normal. Discovered in 1930 it was the first acute phase protein to be recognised, but despite being studied for almost sixty years [McCarty, 1982] its functions are not known with certainty. Many possible roles have been suggested and these are discussed below.
TABLE 1. THE ACUTE PHASE REACTANTS IN MAN.

a) Concentration rises by greater than 10 fold
   C reactive protein
   serum amyloid A

b) Concentration rises by 1-10 fold
   alpha$_1$-acid glycoprotein
   alpha$_1$-antitrypsin
   alpha$_1$-antichymotrypsin
   fibrinogen
   haptoglobin

c) Concentration rises less than 100%
   caeruloplasmin
   C3

d) Concentration falls ("negative acute phase reactants")
   albumin
   transferrin
   prealbumin
History

C-reactive protein was discovered by William Tillet and Thomas Frances working in the laboratory of O T Avery at the Rockefeller Institute in New York [Tillet and Francis, 1930], who were looking at soluble components of the pneumococcal cell wall in an attempt to produce a type-specific antiserum. By repeated freezing, thawing, acidifying and boiling, they isolated a carbohydrate moiety from the bacterial cell wall which they called Fraction C [Tillet et al, 1930], probably because of its analogy to Lancefield's C substance from the streptococcus described a few years earlier, also in the Rockefeller Institute. They added fraction C to sera collected at frequent intervals from patients suffering from pneumococcal pneumonia to see if a precipitation reaction would occur. The observed result was unexpected in that precipitation was greatest in the samples taken at the height of the disease whereas samples taken in the recovery phase produced no precipitate as would have been expected if this had been a classical antibody/antigen reaction.

The explanation of this phenomenon and the nature of the 'C-reactive substance' were made clear only slowly over the next ten years by further work in the Rockefeller laboratories under the direction of Avery. Tillet and Frances had themselves noted the same response in the serum of patients with endocarditis and other infections [Tillet and Frances, 1930], and so realised that the
reaction was not specific to sera from patients infected with the pneumococcus. They thought that gram positive cocci were possibly the common link, but it was soon shown that the reaction occurred with other bacterial infections including Salmonella typhi and E. coli [Ash, 1933]. Attention then turned to other species and it was found that serum from infected monkeys also produced the same precipitation reaction but serum from rabbits and mice did not [Abernethy, 1937], although these too were later shown to produce CRP.

Experiments characterising the chemical and immunological properties of the C-reactive substance were reported in 1941 [Abernethy and Avery, 1941; MacLeod and Avery, 1941a; MacLeod and Avery, 1941b] which established the protein nature of this substance which was now named "C-reactive protein". It was shown that the C reactive substance was inactivated by heating above 65°C and separated with the albumin fraction on ammonium sulphate precipitation. It was further shown that the precipitation reaction with the C carbohydrate was abolished in citrated plasma indicating that the reaction was calcium dependent. Finally an antibody was raised in rabbits to the C reactive substance which produced precipitation reactions at concentrations of C reactive substance of 5 mcg/ml, considerably lower than that required to precipitate with C carbohydrate, but still produced no precipitation with normal sera. It was concluded that the C reactive substance was a protein which was not present in normal sera but appeared in response to bacterial infections.
**Structure**

Human C-reactive protein consists of five identical subunits each with a molecular weight of around 21 kD [Oliveira et al, 1979], arranged into a plate-like structure [Osmand et al, 1977] giving a molecular weight in plasma of around 110,000 to 144,000 [Gotsleiech and Adelman, 1965], which on crystallising may link to form decamers. The complete sequence of 187 amino acids has been determined [Oliveira et al, 1979] and is listed in appendix 1, page 175. The N-terminal residue is modified to pyrrolidone carboxylic acid and there is one disulphide bridge between cysteine residues at positions 36 and 78. No significant sequence homologies have been found with any known human protein [Liu et al, 1982], but some similarities have been noted with the C-gamma-2 site of human IgG which may be the site of complement activation. Significant homologies have however been found with both mammalian and fish CRP, rabbit CRP being more than 90% homologous [Liu et al, 1982]. The significance of these homologies is discussed below.

**Synthesis and turnover**

In man CRP is coded for by a single gene, but in the rabbit two genes exist and in the plaice three [Goldman et al, 1987]. Studies in rhesus monkeys have shown that CRP is synthesised exclusively in the liver [Hurlimann et al, 1965]. Earlier reports of high concentrations of CRP at sites of inflammation [Kushner and Kaplan, 1961; Kushner
et al, 1963], which were interpreted as suggesting that synthesis was occurring locally were refuted by the use of radiolabel incorporation techniques which identified sites of synthesis as opposed to sites of concentration.

Three pieces of evidence show that the increased levels of plasma CRP during the acute phase response are due to new synthesis rather than release of preformed CRP. Firstly the finding of low or undetectable levels of CRP in normal liver by immunohistochemical staining [Kushner and Feldman, 1978]; secondly, the lag phase from the initiating stimulus such as surgical operation to the appearance of increased plasma levels of CRP of around 6 hours consistent with the time required to initiate protein synthesis [Colley et al, 1983]; Thirdly, rising plasma levels are associated with increased transcription of mRNA for several acute phase proteins [Goldberger et al, 1987].

All hepatocytes can synthesise CRP but synthesis begins first in periportal cells and spreads by degree to centrilobular areas, [Kushner and Feldman, 1978] which is in keeping with the stimulus to production being blood borne [Macintyre et al, 1982].

The half life of CRP in plasma is about 6 hours in the rabbit [Chelladurai et al, 1983], and this is not affected by acute phase stimulation. The fate of CRP lost from the circulation is not known for certain but would appear to be consumed principally at the sites of inflammation where it is known to aggregate.
Function

Although there have been a number of suggestions for a useful biological function, the exact role of CRP is not yet certain. However, two pieces of evidence suggest that CRP has a fundamentally important function and is neither an artefact nor an evolutionary remnant of little biological use. Firstly, there is the proportion of the protein synthesising capacity of the liver which is diverted to its production in the face of injury and infection, times when maximum efficiency of the body's resources are required to ensure survival of the organism. It has been estimated that up to 40% of all protein synthesis in the liver may be directed to the production of acute phase proteins [Whicher and Dieppe, 1985]. Secondly, there is the length of time through which the structure of this protein has survived the evolutionary process without significant modification amounting to over 300 million years (see below).

The most widely accepted functions for CRP are related to the binding affinities which have been discovered, and the activation of the complement system which binding induces.

The initial finding of the binding to the C carbohydrate of the pneumococcus was further investigated to ascertain which component of this complex molecule was the actual binding site. The C carbohydrate is a heteropolymer of N-acetyl-galactosamine, murein, ribitol phosphate, choline and diaminotrideoxyhexose [Liu and Gotschlich, 1967; Gotschlich and Liu, 1967; Brundish and
Baddily, 1968; Tomasz, 1967; Distler et al, 1966], and the binding site of greatest affinity is with the phosphorylcholine component [Volanakis and Kaplan, 1971] which has a Kd of $1.6 \times 10^{-5}$M. CRP will also bind with other phosphate monoesters although with lower affinity. Binding requires the presence of calcium and brings about the activation of the complement system [Kaplan and Volanakis, 1974; Volanakis and Kaplan, 1974] by the classical pathway, activating mechanisms of chemotaxis and phagocytosis by macrophages. Phosphoryl choline is widely present in biological lipid membranes and studies with both natural and artificial lipid bilayer membranes have shown that they bind CRP [Narkates and Volanakis, 1982] and that the resulting complement activation renders the membranes permeable to small molecules [Mold et al, 1981].

More recently there has been described a binding affinity for components of the nuclear chromatin, sited at the junction of the polycationic histone protein and the polyanionic DNA, which has a binding coefficient several orders of magnitude greater than that for phosphoryl choline. Binding of CRP to nuclear chromatin also activates complement thus facilitating dissolution of DNA [Robey et al, 1984; Robey et al, 1985]. Nuclear chromatin is one of the most dense and least soluble of intracellular materials and a specific mechanism for its disposal from damaged cells at sites of inflammation would be beneficial to healing and repair.

A possible clinical correlate of this is the
association in certain connective tissue diseases of an impaired acute phase protein response and the presence of circulating antibodies to DNA. It has been speculated that the failure to clear exposed chromatin at sites of inflammation is responsible for the appearance of these antibodies [Robey, 1987].

The role of CRP in opsonising and clearing intact micro-organisms is not clear, particularly in man. Most attention has been paid to the pneumococcus, this being the first noted source of the principle binding ligand, although binding has also been noted to components of other micro-organisms including Proteus morganii, some streptococci and fungi such as Aspergillus sp. [Mold et al, 1982]. Studies on opsonisation and lysis of Streptococci in vitro have been conflicting [Hokama et al, 1962; Williams and Quie, 1968; Kindmark, 1971]. Studies of mice in vivo have shown protection from pneumococcal infection by loading doses of CRP [Mold et al, 1981; Nakayama et al, 1983], but no protection was shown against Salmonella sp. (which do not bind CRP) and interestingly, partial depletion of complement by the injection of cobra venom did not remove the protection.

Thus there is evidence for a functional similarity between CRP and immunoglobulins [James et al, 1983], both proteins binding to unwanted material and bringing about the activation of the complement system to effect their phagocytosis and clearance, but differing in that CRP recognises molecules that are abnormal 'self' rather than foreign. Further support for this analogy is provided for
by the finding of amino acid sequence homology between residues 90-105 of CRP and the C-gamma-2 region of IgG, which is thought to be the binding site for C1q on the IgG molecule [Osmand and Short, 1981], although this homology is thought to be insufficient to suggest a common evolutionary origin [Liu et al, 1982].

**Immune modulation**

Initial studies of interactions between CRP and lymphocytes suggested that this was not a major function of CRP. Although CRP binds to a proportion of peripheral blood lymphocytes this did not appear to have significant influence on proliferation responses, antibody-mediated cytotoxicity, natural killer cell activity or cytokine production by lymphocytes [James et al, 1983; Vetter et al, 1983]. Significant enhancement of cell mediated cytotoxicity however has been described when lymphocytes were incubated in the presence of 200 mg/l CRP which is within the acute phase range in man [Vetter et al, 1986]. More significant perhaps were the effects seen when CRP was first lysed by treatment with neutrophil derived proteases. This produced fragments with potent stimulatory effects on monocyte and neutrophil chemotaxis, superoxide production and interleukin-1 production by monocytes [Robey et al, 1987]. This is supported by the location within these peptides of sequences similar to tuftsin and its analogues, tetra peptides known to have immuno-stimulatory activity.
The stimulation of interleukin-1 production has stimulated the concept of a 'CRP cycle', a positive feedback loop maintaining the availability of CRP as it is consumed at the site of inflammation [Robey and Jones, 1986].

Taken together these finding suggest that CRP probably acts as a mediator of the immune response at a local tissue level, but this function is not as clearly established as its complement activating role.

**Phylogenetic aspects**

Proteins closely analogous to CRP have been found in many species in addition to man and the monkey noted above, including the rabbit [Anderson and McCarty, 1951], chicken, mouse, horse [Patterson and Mora, 1964], dog [Dillman and Coles, 1966], guinea pig [Usui, 1964], plaice [Baldo and Fletcher, 1973] and the horseshoe crab [Robey and Liu, 1981]. The finding of such a protein in a primitive invertebrate species indicates genetic preservation of the molecule for over 300 million years. As stated above, this feature is one of the strongest pieces of evidence of the existence of a fundamental biological role for CRP.

All of these species specific varieties of CRP share the basic property of calcium dependent binding to C polysaccharide, have the same basic pentameric structure (though may form decamers by stacking two subunits) [Baltz et al, 1983], and have similar amino acid sequences [Oliveira et al, 1979, 1980; Pepys et al, 1982; de Beer et
There are however a number of important differences in the properties of the various CRP analogues. In some species, particularly fish, CRP is not an acute phase protein but is present in the plasma at all times with no detectable rise under stress conditions [White et al, 1981]. Mouse CRP behaves similarly to human CRP, being present in trace amounts normally and behaving as an acute reactant but never exceeding 2 mg/l [Siboo and Kulisek, 1978]. In the rat, CRP is normally present at concentrations of around 300 mg/l and increases by a factor of 2-3 under conditions of acute phase stimulation [de Beer et al, 1982].

Another variable property is the ability to activate complement which might be thought to be fundamental to its physiological function if this involves clearance of effete materials from sites of inflammation. Human CRP when bound to its specific ligands activates the classical complement pathway, but this effect is not seen in the mouse [Baltz et al, 1983] or rat [de Beer et al, 1982].

Thus although well preserved structurally, it is not clear if the function of CRP in these various species remains the same or whether different functions have evolved without major changes of structure.

**Normal levels and the response to injury**

The availability of more sensitive methods of measurement using radioimmunoassay in the 1970's showed
that CRP was in fact present in the plasma of normal individuals, but at low concentrations. Two studies of healthy subjects have shown median levels of 580 and 800 mcg/l [Claus et al, 1976; Shine et al, 1981], with levels as low as 70 mcg/l.

The effect of injury on the plasma concentration of CRP has been best studied following elective surgical operation. The concentration of CRP begins to rise after a lag phase of about 6 hours, reaches a peak at about 48 hours and then, in the absence of complications falls gradually to normal over 14 - 21 days [Rapport et al, 1957; Crockson et al, 1966; Werner and Odenthal, 1967; Aronson et al, 1972; Fischer et al, 1976; Colley et al, 1983]. Peak levels reached depended on the degree of surgical trauma inflicted, though there is controversy over what degree of trauma is required to obtain maximal stimulation. One study [Colley et al, 1983] showed a greater rise in plasma CRP following herniorrhaphy than the Trendelenburg operation for varicose veins with peaks of approximately 110 and 15 mg/l respectively. However, they found no greater rise in CRP levels with greater degrees of surgical trauma as represented by cholecystectomy, hemicolecctomy and hip replacement, but the numbers of these other operations were relatively small. It was suggested that there existed a maximal rate of synthesis after uncomplicated surgical procedures that was stimulated by relatively minor degrees of trauma such as herniorrhaphy. They did note however a marked spread of peak values within the apparently homogeneous
herniorrhaphy group ranging from 38 to 196 mg/l.

This finding of a maximal level of CRP stimulated by herniorrhaphy is at odds with the findings of another group [Dominioni et al, 1980] who found a correlation between peak CRP levels and degree of surgical trauma extending to more major procedures. However their procedures other than "excision of melanoma" might all be regarded as major (subtotal gastrectomy, total gastrectomy and abdomino-perineal excision of rectum) and the spread of values for peak CRP after these three procedures was not wide and not statistically significant, though this was not stated. Their data could therefore be consistent with the hypothesis that surgical trauma produces a maximal peak of CRP following relatively minor procedures.

Clinical applications of the measurement of CRP

The acute phase plasma protein response is found in nearly all inflammatory states whether infective or non infective. The presence of CRP in the acute phase of pneumococcal and other bacterial infections is of historical significance but a similar rise is seen also in fungal, parasitic and viral infections, including a modest rise during the common cold [Whicher et al, 1985], and a variety of non infective inflammatory states.

From this three clinical applications of CRP measurement have been identified [Pepys and Baltz, 1983]. Firstly as a screening tool for organic disease, secondly as a measure of disease activity in infective,
inflammatory, malignant and necrotic diseases, and thirdly as a detector of intercurrent infection.

The use of CRP as a screening tool and an indicator of disease activity is comparable to the use of traditional indices such as white cell count, erythrocyte sedimentation rate (ESR) and plasma viscosity and has found clinical application in a variety of diseases including rheumatoid arthritis [McConkey et al, 1972], Crohn's disease [Fagan et al, 1982], and acute pancreatitis [Mayer et al, 1984]. In rheumatoid disease in particular CRP levels have been used to monitor not only disease activity but the effectiveness of treatment regimes [McConkey et al, 1979].

Serial measurements of CRP postoperatively beyond the expected peak at 48 hours have shown that a continued rise or the appearance of a second peak in plasma concentration is associated with the development of infective complications [Fischer et al, 1976; Schentag et al, 1984], and it has been proposed that serial measurements of CRP might be useful in the early detection of infective complications, following abdominal and cardiothoracic surgery particularly in those in whom clinical assessment is difficult as in the paralysed, ventilated patient in an intensive care unit. In pancreatitis too, the appearance of a rising level of CRP after the first phase of the disease is a sensitive indicator of the development of complications such as abscess formation and necrosis.

Similarly in conditions not associated with a rise in CRP levels but where the ESR is raised such as systemic
lupus erythematosus and scleroderma and leukaemias, the appearance of a rise in CRP concentration in the plasma may be the best indicator of intercurrent infection [Mackie et al, 1979] and monitor of response to treatment [Schofield et al, 1982].

**SERUM AMYLOID A (SAA)**

Serum amyloid A shares with CRP the features of very low normal concentration and a rapid, early rise of many hundred fold during the acute phase response, but far less is known about it CRP. It was discovered much more recently and has been less intensively studied partly on account of the difficulty measuring it due to its binding properties.

**Structure and synthesis**

Serum amyloid A is a non glycosylated protein of molecular weight 180,000 D [Levin et al, 1973] composed of subunits of approximately 12,000 D. The amino acid sequence has been determined [Benditt et al, 1982]. Synthesised in the liver [Pepys and Baltz, 1983], it is present in plasma almost totally bound to high density lipoprotein (HDL) as an apoprotein. Present in small amounts in health the plasma concentration increases by several thousand fold during acute phase stimulation [de Beer et al, 1982], from around 1-30 mg/l [Chambers and Whicher, 1983] to as much as 5 g/l at maximal stimulation when it may constitute as much as 50% of the protein
content of HDL [Benditt et al, 1982]. Peak plasma levels occur within 6 - 10 hours of the noxious stimulus, earlier than peak levels of CRP. SAA is one of the components of amyloid deposited in certain organs in a number of chronic illnesses, however the relation between persistently elevated plasma SAA levels and the development of amyloidosis is not straightforward. Nevertheless, there is some evidence that those diseases associated with amyloidosis such as rheumatoid arthritis and Crohn's disease do produce higher and more prolonged peaks of plasma SAA concentration [Pepys and Baltz, 1983].

Function

The function of SAA is even less clear than that of CRP. Because of its association with HDL and in particular HDL₃ a subset of particles rapidly cleared from plasma it has been proposed [Whicher, 1984] that its function may be to help clear cholesterol from lipid laden macrophages emanating from sites of acute and chronic inflammation with their consequent cellular destruction.

Clinical use of the measurement of serum amyloid A

The difficulty in measuring plasma SAA due to its binding to HDL particles has reduced its usefulness as a marker of the acute phase response when compared to CRP. Commercial antisera to SAA for immunological assay were not available as recently as 1984 [Whicher and Dieppe, 1985].

Another factor limiting its usefulness is the extreme
sensitivity of SAA, which is elevated by stimuli as mild as the common cold and other minor viral infections [Whicher et al, 1985; Sarov, 1982]. For these reasons measurement of SAA has found little use in clinical practice.

**ALPHA\(_1\)-ANTITRYSIN (AAT)**

AAT is a 45,000 D glycosylated protein with protease inhibitory activity [Koj, 1974]. Synthesised exclusively in the liver it has a normal plasma concentration of approximately 1.6 - 3.4 g/l, which rises to approximately twice normal after an isolated stimulus such as a surgical operation, reaching a peak on the third day [Werner and Odenthal, 1967]. The half life in plasma is 3.9 days [Kueppers and Fallat, 1969]. AAT binds with the neutral proteases from leukocyte granules namely collagenase and elastase [Whicher, 1984] in addition to providing approximately 90% of circulating antitrypsin activity [Koj, 1974].

Three functions have been proposed for AAT [Whicher, 1984]. Firstly it acts as an inhibitor of excessive protease activity at sites of inflammation to prevent activated proteases being released into the general circulation; secondly, it inhibits proteases acting on the activation of mediator peptides such as the kallikrein and complement systems, thus participating in the control mechanisms of these systems; and thirdly it protects newly synthesised structural proteins laid down at sites of
repair [Davies et al, 1982].

There is a specific deficiency syndrome resulting from the production of two abnormal genetic variants of AAT, which may be heterozygous or homozygous [Davies, 1974]. The abnormal protein inhibits its own release into the plasma and AAT laden granules may be seen on histological examination of the liver of affected individuals. Affected individuals are liable to pulmonary emphysema and hepatic cirrhosis, homozygotes usually being affected in early life.

Alpha₁-antitrypsin has not been adopted as a clinical tool for use in monitoring the acute phase response on account of the variable rate of consumption which it undergoes in chronic vasculitic diseases making plasma levels inconsistent and not directly related to rates of synthesis [Whicher and Dieppe, 1985].

**ALPHA₁-ACID GLYCOPROTEIN (AAG)**

Alpha-1 acid glycoprotein, also known as orosomucoid, is a heavily glycosylated (carbohydrate content 41%) protein of molecular weight 45,000 D. The amino acid sequence is known and the carbohydrate component well characterised [Arnaud and Gianazza, 1982].

The normal plasma concentration is between 0.5 and 1.4 g/l which may rise to 3 g/l during acute phase stimulation [Whicher and Dieppe, 1985], peaking between 72 and 96 hours after initial stimulation such as operation [Fischer et al, 1976; Colley et al, 1983; Crockson et al, 1966]. Its half life in plasma is about 5 days [Weisman et
In contrast to its structure, the function of AAG is little understood though certain observations have prompted suggestions. It has significant sequence homology with immunoglobulins [Ikenaka, 1972], is expressed on the surface of some lymphocytes [Gahmberg and Andersson, 1978] and the purified protein can inhibit T cells [Chiu et al, 1977], implying that AAG may have a role in immunomodulation.

AAG also promotes fibroblast growth and binds to newly formed collagen and thus may also have a role in the repair process after injury [Arnaud and Gianazza, 1982]. Its long half life and slow response time has not rendered it of use clinically as a monitor of the acute phase response.
2. INTERLEUKIN-1 AND THE ACUTE PHASE REACTION

The initiating event in the acute phase response is the aggregation of macrophages of the mononuclear series at the site of injury or inflammation and the release of numerous polypeptide cytokines. The release of these cytokines is initiated by contact with substances released from micro-organisms and inflamed and traumatised tissue. Of the known specific stimulants the most potent is the lipopolysaccharide known as endotoxin, released from the cell wall of effete bacteria. The list of released cytokines known to be active in the acute phase response has grown considerably in the last few years. As recently as 1984 it was thought that interleukin-1 was the common mediator for all the acute phase phenomena and a simple unified model was proposed [Dinarello, 1984b]. It is now realised that there are several different mediators including interleukin-1, interleukin-6 (interferon-beta₂, hepatocyte stimulating factor), interferon-gamma, tumour necrosis factor-alpha and tumour necrosis factor-beta (cachexin). Furthermore it is now known that there are species differences in the various effects of these cytokines and different spectra of acute phase proteins are stimulated by each cytokine. The role of interleukin-1, which is the best understood and described cytokine is described below.
Interleukin-1 (IL-1) is the name given to a protein formerly known by a variety of names, including endogenous pyrogen (EP), leukocyte endogenous mediator (LEM) and lymphocyte activating factor (LAF). Steadily accumulating evidence, reviewed by Dinarello [Dinarello, 1984a], that these molecules had similar molecular weights, kinetics of action, iso-electric points and cells of origin culminated in the proposal at an international meeting of cell biologists in 1979 [Aarden et al, 1979] that these proteins were identical. The name interleukin-1 was coined to emphasise the role of this protein as a messenger between leukocytes. At the same meeting the name interleukin-2 was given to a polypeptide formerly known, amongst other names, as thymocyte mitogenic factor, and the interleukin family was born. Currently there are six members.

Structure and synthesis

Interleukin-1 is a 17,000 Dalton peptide with two distinct subspecies designated IL-1 alpha and IL-1 beta, the latter being the predominant form in man.

Interleukin-1 (of either form) is synthesised as a precursor protein with a molecular weight of approximately 33,000 which is cleaved intracellularly removing the N-terminal end to produce the active form before release into plasma [Giri et al, 1985]. Further cleavage takes place in the plasma and the resulting fragments of approximately 4.3 kD retain biological activity. Cleavage
to this smaller fragment may be essential for some functions, including release of amino acids from skeletal muscle. For this reason the name "proteolysis inducing factor" has been given to the 4.3 kD fragment [Clowes et al, 1983].

The complete amino acid sequences have been determined (appendix 2), by amino acid analysis of the purified protein for IL-1 alpha [Cameron et al, 1985] and by isolation and sequencing of the corresponding DNA for both IL-1 alpha [Gubler et al, 1986] and IL-1 beta [Auron et al, 1984]. Both have been cloned by recombinant DNA techniques. The two forms may be distinguished by their iso-electric focusing points, the pI of IL-1 alpha being approximately 5 and that of IL-1 beta 7.

Despite similar biological activity of IL-1 alpha and beta only four small areas of homogeneity exist designated A-D [Auron et al, 1985]. Two of these are located in the precursor fragment, leaving two areas C & D near the carboxy terminal end which have been proposed as the active site.

The sequence of murine interleukin has also been determined [Lomedico et al, 1984] and is considerably different from the human form with only 62% homogeneity to IL-1 alpha the pI of which it shares.

**Cells of origin**

Cells of the mononuclear phagocyte series represent quantitatively by far the most important source of
interleukin-1. These cells include circulating monocytes as well as peritoneal macrophages, pulmonary alveolar macrophages, hepatic Kupffer cells and splenic macrophages, and all are capable of producing assayable quantities of interleukin-1 [Dinarello, 1984a], when suitably stimulated. This is consistent with the known role of these cells in inflammatory processes.

However a number of other cells have been demonstrated to produce interleukin-1, including corneal epithelium [Grabner et al, 1982], epidermal keratinocytes [Sauder et al, 1982] and glial cells [Fontana et al, 1982]. The role of interleukin-1 is less clear in these tissues but it probably acts locally rather than systemically [Dinarello, 1984b].

**Stimulation of production**

The list of substances known to cause release of interleukin-1 from macrophages is extensive [Dinarello, 1984a], but the most potent are bacteria and bacterial products, in particular the lipopolysaccharide component of the cell wall of gram negative bacteria known as endotoxin. Pico gram concentrations of endotoxin are capable of stimulating interleukin-1 production *in vitro* [Dinarello et al, 1984]. Another toxin, the exotoxin from *Staphylococcus aureus* implicated in the toxic shock syndrome, is equally as potent [Ikejima et al, 1984]. Intact bacteria are also strong stimulators of interleukin-1 production, with as few as 3 bacteria being required to induce detectable quantities of interleukin-1.
[Butler et al, 1982]. Other stimulators of interleukin-1 production are listed in table 2, page 38 (taken from Dinarello 1984a).

The mechanism of release is not clear although there is some evidence that binding of the stimulating substance to the macrophage cell membrane activates the lipoxygenase enzymes responsible for arachidonic acid synthesis and ultimately prostaglandin formation [Dinarello et al, 1983]. There is no evidence of stored intracellular interleukin-1 and release is prevented by inhibitors of protein synthesis.

Attenuation of the interleukin-1 response to in vitro stimulation of mononuclear cells has been described in a number of clinical situations including protein malnutrition (see section 3). Reduced interleukin-1 production has also been noted in patients with connective tissue diseases including systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis and mixed connective tissue disease [Pollack et al, 1983; Whicher et al, 1986]; with solid tumours [Linker-Israeli et al, 1983; Herman et al, 1984]; and with overwhelming sepsis [Luger et al, 1986].

The reduced production of IL-1 in connective tissue diseases is consistent with the known deficiency of acute phase proteins in these patients and may have clinical relevance to the appearance of certain auto-antibodies in the connective tissue diseases (see section 1). The relationship with malignant disease observed in breast and
**TABLE 2. INDUCERS OF INTERLEUKIN-1 PRODUCTION**

Microorganisms
- Viruses, bacteria, spirochetes, yeasts

Microbial Products
- Endotoxins from gram negative organisms
- Peptidoglycans
- Exotoxins from staphylococci and streptococci
- Yeast polysaccharides

Inflammatory agents
- Bile salts
- Etiocholanolone
- Silica
- Urate crystals

Antigens (via antibody or lymphokine production)
- Microbial antigens (eg. old tuberculin, staphylococcal proteins)
- Non microbial antigens (eg. ovalbumin, penicillin, albumin)
- Alloantigens (in the mixed leukocyte reaction)

Plant lectins
- Phytohaemagglutinin
- Concanavalin A

Lymphokines
- Colony stimulating factor
- Macrophage activating factor

Others
- Bleomycin, muramyl dipeptide, CRP
colon cancer and melanoma in the study by Linker-Israeli was complex, in that production was diminished in both early and advanced tumours but normal in intermediate grades of tumours. The finding of a refractory state in early tumours suggested by the authors is unsubstantiated, since a similar study by Herman and colleagues [Herman et al, 1984] found no such effect in early tumours, but confirmed the attenuating effects of advanced tumours. As is discussed in section 3, the differentiation of effects due to advanced malignancy from those due to malnutrition is often difficult given the frequent association of these two phenomena. This point was not addressed in either of these papers.

Luger and colleagues described a failure of interleukin-1 production in patients who died from severe sepsis as compared with survivors and normal controls [Luger et al, 1986]. This mirrored a similar finding with regard to proteolysis inducing factor reported earlier [Clowes et al, 1985]. It remains an open question whether this is a terminal event of end stage multi-organ failure, or whether the failure of the acute phase reaction is a significant factor contributing to the deaths or both, in a 'vicious spiral' of decline.

**BIOLOGICAL ACTIONS OF INTERLEUKIN-1**

Much of the study of the actions of interleukin-1 has been carried out *in vitro*. Until the recent availability of large quantities of recombinant interleukin-1 the principal source of interleukin-1 for experimental work
was from macrophages cultured in the presence of a suitable stimulant and only limited quantities suitable for cell culture work could be obtained.

Other strategies employed to study IL-1 have been the measurement of plasma interleukin-1 activity (by bioassay) in various clinical states and the reproduction of a biological effect by the transfer of plasma from one animal to another.

**Acute phase protein production**

The earliest reports of the ability of interleukin-1 to stimulate the production of acute phase proteins appeared in 1971, when reports from two groups both demonstrated that injection of LEM into rats produced an elevation of acute phase protein concentrations [Pekarek et al, 1972; Eddington et al, 1971]. Similar responses were also subsequently noted in rabbits [Merriman et al, 1975] and mice [Sipe et al, 1979].

Two problems arose in the interpretation of these observations. Firstly the purity of the stimulating substance was suspect and secondly it was difficult to decide if LEM was acting directly or via a second messenger. The LEM used was a crude extract from stimulated polymorphonuclear leukocytes (but in reality arising from contaminating mononuclear cells present in the same specimen), and probably contained many other substances. However the correlation of acute phase protein stimulation with the pyrogenic effect observed when the
same preparation was injected into animals suggested that it was indeed LEM (or EP) that was the active substance.

The absence of any hormonal influence in this response was later demonstrated, by repeating the experiments in endocrine ablated animals [Wannemacher et al, 1975].

The issue was further resolved by the use of hepatocyte cultures. Hepatocytes cultured from the rat [Koj et al, 1985], and mouse [Baumann et al, 1983] were shown to produce acute phase proteins in response to exogenous IL-1 prepared from conditioned (stimulated) monocytes from the same species. In addition rat and mouse hepatocytes in culture were also stimulated by conditioned medium from human monocytes [Gauldie et al, 1987], and keratinocytes [Baumann et al, 1984]. These experiments eliminated the possibility of a secondary messenger or a second cell type, but the purity of the interleukin-1 preparations remained a problem, which was only resolved with the availability of pure interleukin-1 prepared by genetic engineering.

The availability of recombinant-DNA IL-1 and other cytokines has revealed new complexities in the mechanisms of acute phase stimulation which had already been suspected from work using fractionated preparations of conditioned monocyte medium, and has revealed that there are several cytokines capable of stimulating production of acute phase proteins, that they have differing importance in different species and that they produce a different spectrum of proteins in each species. [Kushner, 1987]
The ability of human IL-1 to induce synthesis of the complete array of acute phase proteins was brought into question by studies using human hepatoma lines [Darlington et al, 1986; Baumann et al, 1987] but when tested on murine cell lines the full spectrum of activity was found [Ramadori et al, 1985]. Unfortunately it has not yet proved possible to culture normal human hepatic cells hence complete elucidation of the controlling mechanisms of acute phase protein synthesis in man may be some way off.

Muscle proteolysis

In order to synthesise new protein in the liver a source of amino acids is required and this may be provided by the breakdown of skeletal muscle that accompanies trauma and sepsis. IL-1 was shown to be capable of inducing proteolysis in skeletal muscle by experiments involving the incubation of muscle in the presence of IL-1 in vitro [Baracos et al, 1983]. At the same time another group from Boston demonstrated the same activity in a peptide isolated from the plasma of patients with trauma and sepsis [Clowes et al, 1983]. This peptide which they named 'proteolysis inducing factor' (PIF) had a molecular weight of 4,300 D as opposed to the 15,000 D of IL-1. Further work however showed this new peptide to be a cleavage fragment of IL-1 [Dinarello et al, 1984]. This suggested a consistent mechanism whereby IL-1 not only promoted synthesis of acute phase proteins in the
liver but also provided the substrate for their synthesis by the breakdown of skeletal muscle.

**Fever**

The role of IL-1 in the genesis of fever has been long established as witnessed by the name endogenous pyrogen (EP) one of the former names for IL-1 [Atkins and Wood, 1955]. Regardless of the initiating cause fever is mediated by the release of IL-1 from macrophages which then acts on the thermoregulatory centre in the hypothalamus by a mechanism involving prostaglandins of the E series [Dinarello and Wolff, 1982]. This last fact explains the efficacy of cyclooxygenase inhibitors such as aspirin in reducing body temperature during fever.

**Immune modulation**

Amongst the former names of IL-1 was lymphocyte activating factor on account of its role in the proliferation of both T and B lymphocytes. The activation of T-cells appears to depend on the direct action of IL-1 on T helper cells to induce the synthesis of IL-2 which is the major controlling factor in the proliferation of most T cell subsets [Mizel, 1982].

Less well characterised is the effect on B cell proliferation. Antibody production is noted to be enhanced in the presence of IL-1 [Dinarello, 1984b]. Some of the increase in immunoglobulin synthesis may be direct [Wood and Cameron, 1976] and some via the indirect action of IL-2 [Farrar and Koopman, 1979].
Neutrophil release from the bone marrow is also stimulated by IL-1. The mechanism appears to be a direct action [Dinarello, 1984a] increasing the peripheral leukocyte count within 30 minutes of injection of IL-1 in the rat [Kampschmidt and Upchurch, 1980].
3. THE EFFECT OF MALNUTRITION AND REFEEDING IN THE SURGICAL PATIENT

Malnutrition means bad nutrition and may exist in many forms. As well as the spectre of global protein energy deprivation familiar in areas of famine it also includes the effects of overnutrition, with consequent obesity, and problems associated with imbalances of nutrition. Imbalances include deficiencies of specific nutrients such as vitamins and minerals causing particular deficiency syndromes, and protein deprivation in the presence of adequate calorie intake which causes kwashiorkor characterised by a swollen abdomen and peripheral oedema.

While recognising the importance of these various forms of malnutrition which may have their own implications for surgical practice it is with the problem of global undernutrition that I am principally concerned and it is to this that I will refer in using the term 'malnutrition'.

In man, as in other animals, deprivation of food produces metabolic adjustments in order to maintain essential bodily functions. The most obvious change is that the body must obtain both energy and substrates for biosynthesis from stored reserves. Energy is stored in the form of glycogen in liver and muscle, protein, principally in skeletal muscle, and fat in adipose tissue. Amino acids for protein synthesis and essential gluconeogenesis are
derived principally from the skeletal muscle mass. Glycogen is present in relatively small amounts in the liver and skeletal muscle and is therefore strictly limited to short term utilisation being completely exhausted within hours.

The most noticeable and early effect of malnutrition is the wasting of muscle and adipose tissues as these are broken down and mobilised for utilisation elsewhere. In order to limit the exhaustion of stored reserves it is necessary to reduce consumption of energy and substrates. It is the adoption of a 'siege economy' metabolic state with reduction in activity and capacity of protective physiological mechanisms which is the basis for the harmful effects of malnutrition long before total exhaustion of supply leads to organ failure and death.

Although the concept of a 'siege economy' is a rather teleological view of the physiological changes which accompany malnutrition it helps I think to establish a concept of subclinical malnutrition which does not depend on the presence of gross cachexia to be manifest and indeed may be present in the grossly obese.

In gastrointestinal disease malnutrition arises for a number of reasons. Food intake may be reduced by anorexia and nausea which accompanies many gastrointestinal diseases. Obstructions of the gastrointestinal tract such as oesophageal stricture and pyloric stenosis may also prevent food intake and food may be lost by vomiting. Even when ingested, food may not be adequately digested or absorbed due to diseases of the pancreas, biliary tree and
small bowel. Therefore gastrointestinal disease by its very nature in affecting the organs responsible for intake and assimilation of food has a high probability of being accompanied by some degree of malnutrition.

Weight loss and malignancy are commonly associated but it is not always certain that this weight loss is due to malnutrition in the sense of inadequate availability of nutrients. Despite much investigation the mechanism or mechanisms remain obscure [Theologides, 1974]. The factors outlined above certainly play a part but there are patients in whom weight loss occurs despite adequate food intake and no measurable deficiency in digestion and absorption. Attempts at an explanation of this phenomenon have looked at the metabolic rate of tumour tissue itself, effects on the host metabolic rate mediated by hormones and other mediators, and 'futile cycles' in adipose tissue [Theologides, 1974]. No single satisfactory explanation has however been arrived at.

Any study which defines malnutrition by weight loss and includes cancer bearing patients must take this into account since if inadequate intake is not a prime causation of weight loss then provision of extra food by whatever means will not have any beneficial effect. In studies of the therapeutic effect of food provision, the benefit induced in patients whose weight loss is indeed due to failure of food supply may be masked by those whose weight loss is not.
THE EFFECTS OF MALNUTRITION IN SURGICAL PATIENTS

Almost no physiological process is ultimately immune from the effects of malnutrition but I will discuss here firstly those abnormalities that are in common use as methods of assessment of malnutrition and secondly those which are likely to have a direct bearing on the frequency of postoperative complications by diminishing the mechanisms of repair and resistance to infection.

THE ASSESSMENT OF NUTRITIONAL STATUS

Although it is easy to think of malnutrition in conceptual terms as outlined above it is much more difficult to produce a definition which can be applied to an individual. The assessment of an individual's 'nutritional status' remains one of the most difficult tasks for the clinician and researcher alike. Many indices have been proposed as markers of nutritional status but all have limitations to a greater or lesser degree and there is no single 'gold standard' to which one may refer.

In surgical practice there is an unfortunate tendency to make the assumption that because malnutrition is a cause of post operative complications a good marker of malnutrition, therefore, is one which has a strong association with the occurrence of postoperative complications. This argument has tended to lead to the acceptance of 'nutritional markers' and 'prognostic nutritional scores' which may have little true relationship to nutrition per se but are instead really markers of illness reflecting many underlying processes.
Anthropomorphic measurements

Anthropomorphic measurements attempt to estimate the masses of various components of the body composition by direct or indirect means. The commonly estimated values are for body weight, mid arm circumference, skinfold thicknesses in the biceps, triceps, subscapular, and supra-iliac regions. From the skinfold measurements may be derived the lean body mass [Durnin and Womersley, 1974] Less commonly the mass of certain elements such as potassium, nitrogen and carbon may be determined. From measurements of total body potassium (which may be estimated by the counting of emitted radiation from naturally occurring $^{40}$K), and total body nitrogen (which may be estimated by the technique of in vivo neutron activation analysis [Oxby et al, 1980]), may be derived the body cell mass and the mass of skeletal muscle [Burkinshaw et al, 1979].

It is clear that any of these values measured once in a single individual can only tell whether that individual is big or small, fat or thin, and unless they lie at the extreme ends of the ranges of variability carry no information on the nutritional state of that individual whatsoever. To be constitutionally thin is not to be malnourished as is attested by many long distance runners, but it does imply a reduced level of reserves in the event of loss of supply of nutrition which may bring an early impairment in physiological mechanisms.
Anthropomorphic measurements do however have value when applied to populations of patients, who may be assumed to be abnormal if their group values differ from a matched group of the normal population, and when applied serially to an individual when the measured variables change with time.

One exception is provided by the observation that the ratio of total body potassium to total body nitrogen (TBK/TBN) does not change with body size in the normal population [Morgan and Burkinshaw, 1983] but is reduced in patients with rapid weight loss [Cohn et al, 1981; Burkinshaw and Morgan, 1985], probably due to a disproportionate loss of skeletal muscle. This offers the possibility of assessing a patient's nutritional status from a single measurement of elements of body size but this technique lacks sufficient precision and is principally a research tool at present.

**Estimation of plasma proteins**

The estimation of circulating plasma proteins, and in particular albumin, represents an attractive method of assessing malnutrition because of the ease of performance and the apparently simple relationship between substrate supply and synthesis, and hence circulating levels. Serum albumin concentration remains the most commonly cited 'nutritional marker' in the surgical literature.

There are however a number of pitfalls in the use of albumin as a marker of malnutrition.

i) Synthesis of albumin may be reduced by disease states
which affect liver function despite an adequate substrate supply as in hepatitis, cirrhosis, and many forms of malignancy.

ii) In the acute phase response albumin acts as a negative acute phase reactant. Therefore any condition in which the acute phase response is activated including the postoperative state will result in an inhibition of albumin synthesis.

iii) Plasma albumin concentration may be reduced by excess losses from the gut and urinary tract.

iv) Loss to the extravascular space occurs in many disease states due to the leaky capillary syndrome [Jeejeebhoy, 1962].

v) Plasma albumin concentration may be reduced by dilution. Bed rest alone may be associated with a rise in plasma volume of 7% with equal reduction in plasma albumin concentration [Eisenberg, 1963], and many of the disease states under discussion produce a further increase in extracellular and plasma volume.

vi) The long half life in plasma (20 days) means that it responds only slowly to changes in nutritional status produced by disease or nutritional intervention.

vii) The type of malnutrition may affect the degree to which plasma albumin is reduced. Protein deprivation in the face of adequate calorie intake may produce severe reductions in plasma albumin, but in combined protein and energy malnutrition depression of plasma albumin is a later and less marked phenomenon.
It is now quite clear that non nutritional factors especially the changes in vascular volume and loss to the extracellular space are far more important determinants of albumin concentration and that therefore as a method of assessing nutrition in the surgical patient population, many of whom will be suffering from malignant and/or inflammatory processes albumin has limited value.

Other transport proteins such as transferrin and prealbumin suffer similar disadvantages except that they have a shorter half life.

Muscle function

The simplest 'function' of skeletal muscle to measure is its strength. As skeletal muscle wastes, its strength which varies with cross sectional area falls regardless of any metabolic changes which take place at a cellular level. A convenient group of muscles to measure in this way are the forearm muscles responsible for grip strength which can be measured with a simple dynamometer calibrated in kilograms force. However this method suffers from the disadvantage that grip strength varies with many factors other than nutrition, including age, sex, occupation and the presence of intrinsic disease of the hand and forearm and so, like anthropomorphic data, a single measurement can only say whether an individual is strong or weak though the malnourished as a group are likely to be weaker than average. In addition there is a considerable day to day variation in measured values to up to 30% [personal data]. For all these reasons an individual reading must be
interpreted with caution.

An alternative approach is to measure characteristics of muscle contractility which are dependent on energy supply rather than muscle bulk. A method has been described for measuring the response of the adductor pollicis muscle to an electrical stimulus applied to the ulnar wrist at the nerve [Edwards et al, 1977]. This muscle was chosen for ease of recording after it had been shown to behave in a similar fashion to other larger muscle groups. The variables measured are the maximum relaxation rate (MRR) and the force frequency relationship (FFR). The force frequency measurement compares the force generated at a stimulation frequency of 20Hz to that at 100Hz. As the relaxation rate decreases, the closer the two figures become. Because it is dependent on metabolic changes which can occur before any visible wasting is noted this technique has been advocated as a sensitive index of subclinical malnutrition [Russell et al, 1983].

Jeejeebhoy has demonstrated that patients with gastrointestinal disease who were malnourished as assessed by recent weight loss and clinical assessment have significantly different values for both MRR and FFR to a matched group of normally nourished patients and these parameters had a significant negative correlation with the degree of weight lost [Lopes et al, 1982]. Nutritional repletion by intravenous feeding was shown to return muscle function towards normal in this study and also in patients with anorexia nervosa [Russell et al, 1983].
Immunological function

Epidemiological studies in third world countries have repeatedly shown a relationship between protein energy malnutrition and the incidence of a number of infectious diseases [Tomkins, 1986], but the mechanisms of this association remain poorly understood, and there is a great deal of conflicting evidence in the literature. Most workers have not shown any difference in circulating antibody levels between normal and malnourished individuals [Keet and Thom 1969; Najjar et al, 1969], and tests of cell mediated immunity in vitro have been shown not to be related to protein energy malnutrition in hospital patients [Dowd et al, 1986]. The use of delayed cutaneous hypersensitivity to test antigens has aroused a great deal of controversy. Several investigators have claimed that anergy to test antigens results from protein energy malnutrition and is restored by intravenous nutrition [Meakins et al, 1977; Kaminski et al, 1985]. However delayed cutaneous hypersensitivity is affected by the presence of malignancy and infection which many of the malnourished patients have suffered from and unravelling these is complex [Meakins et al, 1977]. Indeed in a major review of the subject [Twomey et al, 1982] it was concluded that there was no hard evidence to associate skin anergy to malnutrition because of the failure of the experimental designs to take into account other factors which influence the reaction.

That malnutrition is associated with increased liability to infection in the community and in hospital
patients seems clear from epidemiological data, but the mechanisms for this remain obscure and the use of immunological tests currently available as tests of malnutrition or predictors of postoperative outcome is unproven.

**Wound healing**

An association between nutrition and wound healing has been recognised for at least 70 years [Clark, 1919]. Most observers have concentrated on either the association between hypoproteinaemia and the incidence of wound disruption [Rhoades J E, *et al*, 1942; Efron, 1965] or the effect of specific nutritional deficiencies, particularly vitamin C and zinc, on wound healing either in patients or experimental animals [Hunt, 1941]. For reasons outlined above neither of these may be appropriate indicators of malnutrition in patients. A recent series of experiments measuring "wound healing response" by the deposition of hydroxyproline in an implanted porous tube has suggested that even minor degrees of malnutrition are associated with impaired wound healing response and that this can be reversed by adequate food intake either by the oral or intravenous routes without the need to fully restore body reserves or return plasma protein values to normal [Haydock and Hill, 1986; Haydock and Hill, 1987; Windsor *et al*, 1988].

The importance of wound healing in the genesis of morbidity and mortality lies not only in the healing of
the external wound but particularly in the healing of intestinal anastomoses, whose disruption is invariably associated with serious consequences.

**MALIGNUTRITION AND THE ACUTE PHASE RESPONSE**

No study in man has looked directly at the effect of nutrition on the production of acute phase proteins in response to a stimulus such as an operation [Dionigi et al, 1983]. Nutrition has been studied in relation to the acute phase response in three areas; firstly the effect of nutrition on 'background' levels of acute phase proteins; secondly the effect in animals of protein deprivation on acute phase protein synthesis; and thirdly the effect of malnutrition on the ability of stimulated monocytes to produce interleukin-1 in both man and animals.

A study of the effect of seven days of preoperative intravenous nutrition in 20 patients with predominantly malignant gastrointestinal conditions [Coombes et al, 1986] showed no statistically significant difference in the pre-operative plasma concentration of C-reactive protein. They noted however that those patients who did experience a rise in CRP also showed a reduction in the plasma concentration of other plasma proteins more usually accepted as markers of nutritional status including thyroxine binding prealbumin, transferrin and retinol binding protein suggesting that the rise in plasma CRP concentration was not related to any improvement in nutritional status but to an activation of the acute phase response. It also correspondingly suggested that a fall in
the carrier protein concentrations may also have been due to factors other than the nutritional intervention by intravenous feeding. The use of background levels, that is the plasma concentration in an individual who is not subject to an overt source of stimulus of the acute phase response, is unsuitable as a means of determining the degree to which the acute phase response is modulated by nutritional status since it is much more sensitive to stimulation by subclinical infections and minor trauma than by the relatively small contribution of nutrition. It is therefore necessary to assess the acute phase response to a consistent or supramaximal stimulation in order to observe the influence of nutrition.

Animal work

It has been demonstrated that protein starved experimental rats have an impaired ability to produce acute phase proteins. Rats fed on a protein free but otherwise normal diet for eighteen days produced lower plasma levels of acute phase globulins in response to injury (the implantation of a fixed volume of foreign material) than normally fed rats [Neuhaus et al, 1963]. Similarly, isolated perfused livers from rats fed protein deficient diets for six days released less alpha-1 acid glycoprotein than livers from normally fed rats [Miller and John, 1970].
Nutrition and interleukin-1 production

The effect of nutritional status on interleukin-1 production has been examined in both animal models and human studies.

Work done on the rabbit [Hoffman-Goetz and Kluger, 1979] showed that the febrile response to inoculation with a pathogenic gram negative bacterium was attenuated in protein deprived animals. However when the animals were injected with endogenous pyrogen (EP) raised from leukocytes of healthy donor animals, the control and protein deprived animals mounted a similar febrile response. From this it was concluded that the attenuation of the fever was caused by failure of synthesis of EP rather than an inability to react to it in an appropriate manner. A similar later study carried out in rats [Bradley et al, 1987] found the converse, namely that dietary protein deprivation did produce an attenuated febrile response to an injection of purified interleukin-1 as well as to injections of endotoxin.

Follow up studies were carried out in rabbits [Hoffman-Goetz et al, 1981] by the injection of EP raised from the leukocytes of malnourished patients before and after 7 days of intravenous nutrition. Patients were divided into those with protein energy malnutrition (marasmus) and those with predominantly protein deprivation (kwashiorkor). The degree of fever produced increased after nutritional repletion, that in the kwashiorkor group from the lowest level. When a similar protocol was employed in rats [Keenan et al, 1982] the
results were less clear cut but the ability of the patients' macrophages to produce leukocyte endogenous mediator in vitro as assayed by the leukocyte response following injection into the rat was also related to dietary protein intake. These studies suggest that the rabbit and the rat may behave differently or the differences in the experimental methods may account for the slightly differing findings in the two animals.

Attenuation of the capacity of monocytes to produce interleukin was also found in a study of malnourished children in India [Bhaskaram and Sivakumar, 1986]. Macrophages from children with severe kwashiorkor or marasmus and controls were stimulated in vitro to produce interleukin-1 which was then assayed in a conventional thymocyte assay. Interleukin-1 production from the cells of malnourished children was significantly less than controls.

It is therefore clear that the production of acute phase proteins may be reduced in man through malnutrition either by the reduced production of the mediator interleukin-1 or by the reduced capacity of the liver to synthesise acute phase proteins in response to interleukin-1 and other cytokines. The evidence cited above supports the effect of malnutrition on the first part of this chain but an effect on the second part has not yet been demonstrated.
THE RELEVANCE OF MALNUTRITION

Malnutrition concerns surgeons because they believe that the physiological effects of malnutrition outlined above may compromise the patient's ability to recover both from the disease process and the trauma of a surgical operation and its complications, and because some degree of malnutrition is common amongst surgical patients for reasons which have been discussed above.

THE FREQUENCY OF MALNUTRITION IN THE SURGICAL POPULATION

The frequency with which malnutrition is recognised to occur in a general surgical practice depends on the methods used to assess it. In a survey carried out in a single day on all the surgical patients in a single hospital in the USA [Bistrian et al, 1974], it was estimated that up to 67% of patients were malnourished. Albumin, triceps skinfold thickness and mid arm muscle circumference were determined. Moderate malnutrition was defined as albumin < 35 g/l, or anthropomorphic measurements less than 90% of expected, and severe malnutrition as albumin < 28 g/l or anthropomorphic measurements < 60% of predicted, the normal values being taken from World Health Organisation statistics for the sample population [Jelliffe, 1966]. The proportions in each group were respectively: albumin - 27% moderate, 27% severe; triceps skinfold thickness - 21% moderate, 35% severe; mid arm muscle circumference - 36% moderate, 12% severe.

A similar British study carried out in Leeds [Hill et
al, 1977] surveyed the entire surgical population of a large teaching hospital. Using the criteria of weight loss, arm muscle circumference, plasma albumin, plasma transferrin, haemoglobin and red blood cell vitamin levels they found malnutrition in between 20 and 55% of patients. The greatest incidence of malnutrition was found in patients who were in hospital more than one week after major surgery whose assessment would have been affected by the effects of surgery and sepsis. However in the group of patients measured before surgery between 13 and 39% still had evidence of malnutrition by these criteria.

**EFFECTS OF MALNUTRITION ON POSTOPERATIVE COMPLICATIONS**

As long ago as 1930 it was noted that patients with a significant degree of weight loss had a higher risk of complication following operation for chronic peptic ulcer [Studley, 1930]. More than 20% weight loss was associated with a 10 fold increase in risk of death, from 1 of 28 patients (3.5%) to 6 of 18 patients (33.3%). Since then many other indices of malnutrition have been shown to be associated with an increased risk of postoperative complications.

The next to be studied was the plasma protein level [Rhoads and Alexander, 1955]. A rise in infectious complications from 16.7% to 61.8% was found in patients with a total protein level of less than 63 g/l in a sample of over 100 patients. Albumin alone has also frequently been used as a marker for malnutrition in
assessment of surgical risk [Forse and Shizgal, 1980; Leite et al, 1987], both alone and in conjunction with other nutritional markers in scoring systems, the best known of which is the Prognostic Nutritional Index of Buzby and Mullen [Buzby et al, 1980], incorporating plasma albumin, plasma transferrin, triceps skinfold thickness and a score for cutaneous delayed hypersensitivity. The Prognostic Nutritional Index was shown to have a strong correlation with frequency of complications in a prospective study of 100 patients [Buzby et al, 1980].

Despite the limitations of grip strength as an indicator of malnutrition, it has been claimed that grip strength alone is a sensitive predictor of the risk of postoperative complications [Klidjian et al, 1982]. This group found that grip strength below 85% of expected identified 90% of patients suffering significant postoperative complications in a group of 120 undergoing gastrointestinal surgery. This was remarkable since the normal value used was based on sex alone [Klidjian et al, 1980], with no consideration of age, which has been shown to have a significant influence on grip strength, and other groups have not been able to achieve a similar result.

Thus malnutrition as measured by various criteria has been repeatedly shown to be associated with an increased incidence of postoperative complications in surgical patients.
PREOPERATIVE NUTRITIONAL SUPPORT

If malnutrition is a significant contributor to morbidity after surgery then it would appear logical that nutritional support in the preoperative period should reduce the incidence of complications and this should be demonstrable by properly designed clinical trials. In practice this has proved very difficult despite many attempts and no trial to date has shown convincing evidence of significant benefit derived from the administration of preoperative nutritional support.

This may be a true finding, namely that no benefit is conferred by preoperative nutritional support, which would suggest that the abnormal findings of weight loss, low plasma protein concentrations, poor muscle function etc are produced principally by the disease process and only in a minority or to a minor degree by a deficiency of nutrient supply. Assessment of the efficacy of preoperative nutritional repletion should therefore examine whether the physiological abnormalities attributed to malnutrition can be reversed as well as the clinical outcome following surgery improved and this has already been achieved for most of the systems studied.

Alternatively the lack of observed efficacy of preoperative nutrition may be due to inadequacies in the designs of the trials as has been suggested [Silberman, 1985]. Shortcomings in design have included inadequate length of preoperative feeding [Holter and Fischer, 1977], inadequate numbers of patients [Thompson et al, 1981], failure to make malnutrition an entry criterion to the

- 63 -
study [Heatly et al, 1977], and failure to randomise [Starker et al, 1986]. The most impressive results have come in a study from Philadelphia in the USA [Mullen et al, 1980]. In a group of 154 patients undergoing a major intrathoracic or intra-abdominal resection, the provision of preoperative intravenous nutrition to a high risk group (defined as Prognostic Nutritional Index greater than 50%) reduced postoperative major septic complications 6 fold and mortality 5 fold. This degree of benefit has not been reproduced elsewhere. The selection of patients for feeding was made on undefined clinical grounds and the implication must be that this has introduced a major degree of bias to the results.

Even the trial credited with the best design [Muller et al, 1982], failed to admit patients on the basis of evidence of malnutrition. Nevertheless in 125 patients, undergoing gastrointestinal resection, randomised to intravenous feeding and control groups, the provision of 10 days of preoperative intravenous nutrition produced a reduction in major complications from 19 to 11% and mortality from 11 to 3%, both significant at the 5% level. No difference was seen in the incidence of wound infections or pneumonia.

Against a background of generally negative results these relatively modest benefits have failed to bring about the widespread use of preoperative nutritional support for patients with weight loss or other indications of malnutrition. Definitive answers to the questions of
whether preoperative nutritional support is beneficial, to whom it should be given, in what form and for how long it should be given are clearly urgently needed, but the difficulties of mounting a properly designed trial of adequate size have proved insurmountable to date.
4. AIMS OF THE STUDY

The aim of this study was to investigate a possible relationship between the nutritional status of patients undergoing major gastrointestinal surgery and their ability to mount an acute phase response. As has been set out in the introduction patients who are malnourished have an increased risk of post operative complication and death, and the acute phase response appears to be a mechanism important in the recovery from injury and infection. It has been conjectured that malnutrition may impair the acute phase response and thereby (as one factor amongst many) prejudice the patient's recovery.

The relationship was to be studied in two ways. Firstly (the in vivo study) acute phase plasma proteins would be measured in the early postoperative phase in groups of patients with and without clinical evidence of malnutrition undergoing major surgery, and the influence of nutritional markers on the rise in concentration was looked for.

Since the magnitude of the acute phase response is determined by strong influences other than nutrition (the magnitude of the surgical trauma, the presence of infection etc) it was anticipated that detecting an influence of nutrition in vivo might be difficult.

Therefore secondly, in the in vitro study I set out to devise a model for the study of the acute phase response in vitro which could be used to study the acute phase response of liver under controlled conditions. This
involved the incubation of human liver in the presence of an acute phase stimulant, when only pre-existing patient factors and not factors relating to the operation undergone by the patient would influence the response. This model would then be used to measure synthesis of an acute phase protein (C-reactive protein) under conditions of maximal stimulation, and to correlate production of CRP with patient variables.
PATIENTS AND METHODS
1. PATIENTS

The patients used in these studies were those of the Professorial Surgical Unit of the General Infirmary at Leeds. Four groups of patients were studied;

i) a control group of normally nourished individuals undergoing elective surgery for cholelithiasis or peptic ulcer disease (group 1)

ii) patients undergoing surgery for gastro-oesophageal carcinoma who had lost less than 7.5% of body weight (group 2)

iii) patients as for group ii who had lost more than 7.5% of body weight (group 3)

iv) patients as for group iii who had received seven days of intravenous nutrition prior to operation (group 4).

Data was collected from all subjects to quantify the degree of malnutrition and to assess liver function preoperatively. In the cancer patients data was also collected to quantify the tumour load and the degree of surgical trauma experienced at operation. The data were;

1. Age (years)
2. Sex
3. Weight (kg)
4. Weight loss (%)

Weight loss was calculated as the difference between measured weight and either the previously measured weight where this was available or the patient's recall weight three months before admission. 7.5% represents approximately 1 stone in the average man.
5. Height (cm)
6. Triceps skinfold thickness (mm)
This was measured over the triceps at a point in the arm mid way between the greater tuberosity of the humerus and the olecranon using skinfold callipers (Holtain Ltd, Crymmych, UK).
7. Mid arm circumference (mm)
This was measured at the same point as 6 above.
8. Plasma albumin (g/l)
9. Plasma alkaline phosphatase (IU/l)
10. Plasma aspartate amino transferase (IU/l)
11. Plasma bilirubin (mmol/l)
Items 8-11 were measured by the routine hospital biochemistry service on a sequential multiple analysis computer ('SMAC').

Patients in groups 2,3 and 4 had the following information recorded in addition.
12. Site of tumour, classified as follows;
   1 middle third oesophagus
   2 lower third oesophagus
   3 cardia
   4 body of stomach
   5 gastric antrum
   6 pylorus
   7 duodenum

13. Stage of tumour, classified as follows;
   1 confined to mucosa
   2 not penetrating wall
3 penetrating wall
4 lymph nodes involved
5 distant metastases

14. Operation performed

15. Duration of operation (minutes). This was taken from the anaesthetic record.

16. Complications which occurred prior to discharge from hospital and were detected by the medical staff attending the patients during routine care. No special investigations were carried out to detect complications specifically for the purpose of this study. The complications were defined as follows;

1. Wound infection: The discharge of pus, or of serous fluid with a positive bacteriological culture.
   a) minor; not delaying discharge or recovery
   b) major; delaying discharge or recovery

2. Deep abscess: Operative finding, the discharge of pus from an intra-abdominal or intrathoracic site or ultrasound or computed tomography evidence of a deep collection in association with fever greater than 38.5°C and/or white cell count greater than 15 x 10^6/l.

3. Wound dehiscence: the dehiscence of the deep layers whether or not the skin dehisces.
   a) minor; not requiring resuture
   b) major; requiring resuture.

4. Chest infection: purulent sputum in association with clinical or radiological signs of pulmonary collapse or consolidation.

5. Septicaemia: Positive blood culture in association
with appropriate clinical signs.

6. Anastomotic leak:
   a) radiological
   b) clinical; operative or post mortem finding or the presence of a fistula or the presence of a deep abscess or peritonitis in association with a radiological leak.


8. Pulmonary embolus: diagnosed by ventilation/perfusion isotope scanning or pulmonary angiography.

**INTRAVENOUS NUTRITION**

The solution administered consisted of 1600 kilocalories as glucose (Glucose/electrolyte solution, Boots, Nottingham, UK), 12.5 g of nitrogen as a balanced solution of amino acids (Freamine, Boots, Nottingham, UK), vitamins (Multibionta, Merck, Alton, UK), and minerals. Fat was added as Intralipid 10% solution on the first and last days of the course (Kabi-Vitrum, Uxbridge, UK) only in sufficient quantity to provide essential fatty acids.

Intravenous nutrition was delivered via a tunneled silicone elastomer subclavian central venous catheter (Nutricath, Vygon, Ecouen France) placed specifically for the purpose and used exclusively for intravenous nutrition.

The nutrient solutions were compounded in the aseptic suite of the pharmacy, and were delivered as a
continuous 24 hour infusion using an controlling pump to regulate the rate of flow.

Intravenous nutrition was stopped on the evening of the day before operation and was not recommenced after operation unless a complication occurred, such as an anastomotic leak, which would delay the return to eating or the commencement of enteral nutrition.
2. THE IN VIVO STUDY

In this arm of the study blood samples were collected in the post-operative period in order to measure the concentrations of acute phase proteins. The levels measured in the different patient groups were then compared and correlations sought between acute phase protein concentrations and patient nutritional variables.

Patients from groups 2, 3 and 4 as defined on page 69 were entered into this arm of the project, i.e. those undergoing gastric or oesophageal resection for carcinoma.

Blood was collected at the following times; 7 days and 1 day preoperatively, at the beginning of the operation, and at 12, 24, 36, 48 and 72 hours post operatively. Ten mls of blood was placed in a lithium heparin tube and centrifuged immediately to obtain plasma which was stored at -70°C until assay in batches. The following proteins were measured;

1. C-reactive protein
2. alpha₁-acid glycoprotein
3. alpha₁-antitrypsin
4. transferrin
5. prealbumin

The assays were carried out on an automated immunoassay system [Beckman, Los Angeles, USA] using the principle of laser nephelometry. A measured aliquot of plasma was added to a solution containing excess antibody to the substance to be measured. A laser beam was passed through the liquid and caused to scatter by the resulting
antigen/antibody complexes in solution. The degree of scatter was measured by a photoelectric cell placed at an angle to the direction of travel of the laser beam. The concentration of the test substance was derived by comparison to standard curves obtained by measurements of samples of known concentration.

C-reactive protein was measured at all the time intervals indicated. For the other proteins the 12 and 36 hour samples were omitted because the plasma concentrations of these proteins react more slowly.

All 34 patients had blood samples taken at 0, 24, 48 and 72 hours and 21 had samples taken at both 12 and 36 hours. This was less than the whole study population as it was felt unjustified to wake patients in the night to take blood samples from those who did not have indwelling vascular access. All of these samples were analysed for CRP. The decision to measure the other four proteins was taken after the beginning of the study and before duplicate specimens were kept. The first four patients (study numbers 1,3,5,7) therefore did not have measurements of these proteins, leaving 30 samples for analysis split 17, 8 and 5 into groups 2, 3 and 4 respectively.
3. THE IN VITRO STUDY

In this arm of the study patients in all four groups as defined on page 69 were investigated. Liver was taken by biopsy at the time of operation, cut into thin slices and incubated at 37°C for variable periods and under varying conditions of atmosphere and incubating mechanism. CRP was measured in the medium and in the liver samples at the end of incubation and the synthesis of CRP was compared amongst the four groups and correlations were sought with nutritional variables.

This part of the study was conducted in two phases as the conditions of incubation initially chosen were unable to sustain the liver slices for the duration of the experiment. This had a deleterious effect on the ability to draw conclusions from the experiment.

Liver biopsy

From each subject a wedge biopsy of the left lobe of the liver weighing between 0.24 and 2.16 g was obtained at laparotomy, by a standard surgical technique [McGinn, 1981], immediately after the abdomen was opened. The sample was placed directly into a sterile solution of 0.9% saline stored at 4°C and transported on ice to the laboratory, where it arrived within 5 minutes of removal.

Sample processing

The gross sample was placed on a sterile filter paper on the platter of an electronic balance (Electrobalance,
Cahn Instruments, Cerritos, California, USA.) and the weight noted.

Using sterile instruments, the sample of liver was first trimmed with a scalpel to remove the liver capsule and then cut into strips approximately 0.5 x 1 cm in section removing visible areas of connective tissue as much as possible. The liver specimens were then placed onto a soft grade filter paper to which they adhered and the filter paper was attached by clips to the platter of a McIlwain tissue slicer (Mickle Laboratory Engineering Co, Guildford, UK) with one or two drops of water to help the paper lie flat on the platter. This device [McIlwain and Buddie, 1953] consisted of a slicing arm onto which was mounted a standard double edged razor blade (Wilkinson Sword Ltd, Cramlington, UK) which was made to rise and drop by spring tension onto a platter which was mechanically synchronised to advance by a variable increment in time with the chopping action of the arm. The size of the increment was adjusted by a micrometer and was set to 0.4 mm after experimentation to achieve the thinnest slices which would slice consistently.

Following sectioning the specimen was returned to the iced saline where the slices were teased apart with a fine spatula and a scalpel ready for transfer to the tissue culture dishes.

One to four slices weighing in total between 4 and 73 mg were then transferred to a sterile single use tissue culture dish (Lux 5221, Miles Laboratories Inc.,
Naperville, Illinois, USA) and covered with 5 ml of medium. These were then covered with a lid sitting on lugs which allowed the circulation of gas. The dishes were then placed in an incubator.

**The medium**

The basic medium chosen was Dulbecco's modified Eagles medium (DMEM; for composition see appendix 3). To this was added in a proportion of 1 part to nine parts of medium, either foetal calf serum (FCS) or distilled water. This was done in order to assess the effect of FCS on the preservation of the hepatocytes during incubation (both solutions Gibco Ltd, Paisley, UK). To this was then added varying amounts of recombinant interleukin-1 alpha solution containing 1 U/mcl (Cystron, New Jersey, USA) to give a final concentration of between 1 and 20 U/ml of IL-1, in order to construct a dose response curve. The majority of incubations were carried out in the presence of 10 U/ml IL-1. Antibiotics were also added (see below).

All solutions were mixed aseptically in beakers cleaned in detergent solution, rinsed sequentially in tap water, absolute alcohol and doubly distilled water, before autoclaving at 130°C. Sterile graduated syringes were used to draw up the solutions.

Over a magnetic stirrer and using a magnet cleaned by the same method as the beakers a pH probe was inserted and the pH adjusted to 7.4 by adding 1 M NaOH drop by drop.

The osmolality of the medium including foetal calf serum was 309 mosm/kg.
Incubation

For the first phase of the incubations, the samples were placed in a laboratory incubator (Laboratory Electrical and Engineering Co., Nottingham, UK) at 37°C in an atmosphere of 5% CO₂ and 95% air and 100% humidity for a variable period of between 12 and 96 hours.

For the second phase of the incubations the oxygenation of the samples was increased by suspending the liver slices at the gas liquid interface and changing the gas phase to 95% O₂/5% CO₂ as described by Trowell for the technique of organ culture for less demanding tissues [Trowell, 1959]. This was achieved by placing the slices on a raft of stainless steel mesh (Minimesh 940MM: Expanded Metal Company Ltd, Hartlepool, UK). A strip 2 by 3 cm was bent at the ends to create legs which would raise the 'platform' to the surface of the liquid with 5ml of medium in the dish. The culture dishes were then placed on a perspex carrier specially designed to fit into a McIntosh Fieldes anaerobic jar into which it was placed. This jar is made of cast aluminium with a gas tight lid and has two gas ports passing through the lid one passing via a pipe to the bottom of the jar and one opening directly to the top of the jar. Fifty ml of distilled water was placed in the bottom of the jar to ensure 100% humidity when the jar was sealed.

A mixture of 95% O₂/5% CO₂ (British Oxygen Co. Ltd., London, UK) was passed through the McIntosh Fieldes jar for ten minutes at a flow rate of 5 l/min by connecting
the gas cylinder to the port of the gas jar which opened at the bottom of the jar and venting the other to the atmosphere. The duration of gassing was determined by measuring the oxygen content of the outflow gas using an oxygen analyser (Model 5552 with sensor, model 5555, Hudson Corporation, Temecula, California, USA). The outflow gas reached a steady state within 7 minutes at an oxygen concentration indistinguishable from that of the inflow gas (Figure 2, page 81). A further 3 minutes was allowed as a safety margin for any pockets of slowly mixing gas within the jar.

The jar was then placed in the incubator described above at 37°C.

**Weighing of the slices**

This was performed after incubation in order to reduce the risk of bacterial contamination, to reduce trauma to the unincubated slices and to minimise the processing time that the liver specimen would spend in in a cold dry atmosphere. The liver slices were blotted dry on filter paper and then weighed to the nearest 0.1 mg on an electronic balance (Electrobalance, Cahn Instruments, Cerritos, California, USA.)

**Preparation of samples for intracellular CRP measurement**

Slices for intracellular CRP measurement were transferred from the balance to a ground glass tissue homogeniser to which was added 0.5 ml sodium citrate solution. Citrate was added to prevent CRP binding.
Figure 2. Equilibration of the gas mixture in the McIntosh Fieldes jar at a flow rate of 5 l/min.
excessively to damaged cellular components which might have rendered it 'hidden' to the antibody of the ELISA.
After grinding for two minutes the residue was washed out of the homogeniser and diluted to 5 ml with DMEM.

**Bacterial contamination**

Before each incubation a sample of each component of the medium was sent for quantitative bacterial culture in the routine diagnostic laboratories in the department of microbiology. A 1ml sample of the medium from each dish was also sent after incubation. No antibiotics were added to the medium initially but the results of the cultures in the first two incubations showed almost uniform contamination of the medium by bacteria reported as "mixed environmental organisms" and it was therefore decided to add antibiotics to the incubation medium. These were benzyl penicillin 60 mcg/ml (Glaxo Laboratories Ltd, Greenford, UK) and gentamicin 50 mcg/ml (David Bull Ltd, Warwick, UK). These concentrations were taken from previous work with monolayer hepatocyte cultures and were known not to be toxic to hepatocytes in culture [Koj et al, 1984]. The results of the contaminated incubations were discarded.

**Assay of C-reactive protein**

After incubation 1 ml of the medium from each incubation dish was collected and stored frozen at -70°C before analysis in batches in the department of chemical pathology using an enzyme linked immunosorbent assay
(ELISA).

Fifty microlitre aliquots of whole rabbit antiserum to human CRP (DAKO A073) diluted 1:3650 were bound in excess to a 96 well microtitre plate by overnight incubation (16 hr) in barbitone buffer pH 8.8 at 4°C. The plates were washed 3 times in phosphate buffered saline, pH 7.2, containing 0.05% Tween-20 (PBS-T). Samples of the medium to be measured were then added along with CRP standards in duplicate 50 mcl aliquots which bound to the rabbit antibodies following incubation for 2 hr at 37°C. The standards were diluted in PBS-T containing 10% foetal calf serum, inactivated by heating to 56°C for 30 min (PBS-T-FCS). After washing in PBS-T, 50 mcl aliquots of a second antisera to CRP conjugated to peroxidase were added (DAKO P227), at a dilution of 1:500 and incubated for a further 2 hr at 37°C. This bound quantitatively to the CRP present. The plates were washed twice with PBS-T and once with phosphate/citrate buffer pH 5.0. As a substrate for the enzyme, 100 mcl of a solution containing 34 mg o-phenylenediamine and 50 mcl hydrogen peroxide in 100 ml phosphate/citrate buffer, was then added and allowed to react in the dark for 30 min at 37°C. The reaction was stopped by the addition of 50 mcl of 2 M sulphuric acid. Absorbance at 490 nm was then read in a microelisa minireader, zeroed on control wells containing phenylenediamine only. A calibration curve was drawn from an of absorbance the wells containing the CRP standards and by comparison a concentration in mcg/l was obtained. This was

- 83 -
converted to mcg/mg liver by the following formula:

\[
\text{CRP concentration in medium (mcg/1)} \times 0.005
\]
\[
\text{weight of slice (mg)}
\]

**Assessment of cell viability**

Assessment of the viability of the liver cells after incubation was attempted by three methods; firstly staining with a vital dye, trypan blue, secondly histological assessment by light microscopy and thirdly by determination of intracellular enzyme levels before and after incubation.

i) Trypan blue staining

This was carried out after incubation following trypsin digestion of the slices to obtain single cells from the liver sample. One of the slices from each dish to be tested was washed in a buffered salt solution and then placed in further buffered salt solution containing 10% v/v trypsin solution (2.5%) and this was agitated at 37°C for 30 minutes or until the piece of liver tissue had begun to disintegrate. The solution was then centrifuged for 5 minutes at 1500 rpm and the cells transferred by pipette to a measured microscopic grid for counting of intact and damaged cells.

ii) Histology

After incubation the liver slices were placed in 10% neutral buffered formalin. The fixed tissue was then
prepared for histological examination. The liver was dehydrated by washing with increasing concentrations of ethanol, 'cleared' by washing in xylol, and embedded in paraffin wax. After slicing on a microtome the specimen was dewaxed with xylol and rehydrated with decreasing concentrations of ethanol. The stains employed were haematoxylin and eosin (H & E) for routine examination and periodic acid-Schiff's (PAS) reagent to stain for glycogen. The stained slices were then mounted and examined by an experienced gastrointestinal pathologist who reported on the degree of preservation of the cellular architecture.

iii) Determination of enzyme activity

Three key enzymes in the glycolytic pathway were assayed before and after incubation in 10 liver samples. The enzymes measured were phosphofructokinase, fructose 1,6-diphosphatase and hexokinase. Their position in the glycolytic pathway is shown in appendix 4, page 179. The assays were performed as follows.

Phosphofructokinase (PFK).

Weighed samples of chopped liver were homogenised manually at 0°C in ground glass homogenisers of 100mcl capacity in a 10 fold dilution of a medium containing triethanolamine (50 mmol/l), magnesium chloride (2 mmol/l), ethylenediaminetetraacetic acid (EDTA) (1 mmol/l), dithiothreitol (2 mmol/l), and glycerol (2 mol/l),
adjusted to pH 7.4 with potassium hydroxide. Homogenates were stored on ice and assayed within 60 min. 1mcl of homogenate was added to the reacting medium consisting of Tris/HCl buffer (75 mmol/l) containing magnesium chloride (7 mmol/l), potassium chloride (100 mmol/l), potassium cyanide (1 mmol/l), reduced nicotinamide adenine dinucleotide (NADH) (0.17 mmol/l), adenosine monophosphate (AMP) (2 mmol/l), adenosine triphosphate (ATP) (1 mmol/l), fructose 6-phosphate (1 mmol/l), aldolase (0.64 unit), triose phosphate isomerase (14.3 units), and glyceraldehyde 3-phosphate dehydrogenase (0.5 unit). The oxidation of NADH was measured in samples and control standards without fructose 6-phosphate in a Pye Unicam SP 1800 dual beam spectrophotometer set at 340 nm (Pye, London, UK).

Fructose 1,6-diphosphatase

Ten mcl of tissue homogenate as prepared above was added to a reacting medium consisting of Tris/HCl buffer (50 mmol/l) containing dithiothreitol (2 mmol/l), magnesium sulphate (6 mmol/l), EDTA (1 mmol/l), nicotinamide adenine dinucleotide phosphate (NADP⁺) (0.2 mmol/l), fructose 1,6-biphosphate (1 mmol/l), creatine phosphate (17.2 mmol/l), creatine kinase (12.5 units), myokinase (50 units), glucose 6-phosphate dehydrogenase (0.3 unit), and phosphoglucone isomerase (0.7 unit). The reduction of NADP⁺ was followed at 340 nm against controls without fructose 1,6-biphosphate.
Hexokinase

Ten mcl of tissue homogenate as prepared above was added to a reacting medium consisting of Tris/HCl buffer (75 mmol/l) containing dithiothreitol (2 mmol/l), magnesium chloride (7.5 mmol/l), EDTA (8 mmol/l), potassium chloride (1.5 mmol/l), NADP⁺ (0.4 mmol/l), ATP (2.5 mmol/l), creatine phosphate (10 mmol/l), glucose (1 mmol/l), creatine kinase (12.5 units) and glucose 6-phosphate dehydrogenase (3 units). The reduction of NADP⁺ was followed at 340 nm against controls from which glucose had been omitted. All reagents were obtained from Sigma Chemical Co, Poole, UK, Boehringer Corporation, London, UK and British Drug Houses, Poole, UK.

Enzyme activities are given as micromols of product formed per minute per gram of wet liver.
4. STATISTICAL METHODS

All data was stored and analysed using the database software package dBase III+ (Ashton Tate Corporation, Temecula, USA) on IBM PC compatible microcomputers. Statistical tests were performed using Oxstat, a statistical software package (Oxford Logic, Oxford, UK) on the same machines.

All data was assumed to be non parametric. Distributions were described by median and interquartile range (Q1-Q3). The following tests of statistical significance were employed in the analysis. Correlation was assessed by Spearman's rank order correlation coefficient for non parametric data [Siegel, 1956]. Groups of paired data were compared with the Wilcoxon matched pairs signed ranks test [Wilcoxon, 1945]. The corresponding test for unpaired data was the Mann-Whitney U test [Mann and Whitney, 1947].
The protocol of this study conforms to the standards of the Declaration of Helsinki [World Medical Association, 1964]. Ethical permission for the study was obtained from the Ethical Committee of the General Infirmary at Leeds.

Informed consent was obtained from each patient undergoing liver biopsy after reading the following statement:

"We are interested in studying the effect of poor nutrition on the way in which liver cells help to fight infection. We would like permission to take some blood samples from you after your operation (this should not require any extra needles) and to take a biopsy of your liver at the time of your operation. Although not entirely without risk, we believe this is very small and it will not affect you in any other way nor delay your recovery.

Taking part in this study is entirely voluntary."
RESULTS
1. DETAILS OF THE PATIENTS

Fifty eight patients were recruited into the study in total, 34 into the in vivo study and 40 into the in vitro study (including 14 in both). These were divided into 19 patients with benign disease and no weight loss as controls for the in vitro study (group 1), 18 patients with upper gastrointestinal tumours without weight loss (group 2), fourteen patients with upper gastrointestinal tumours and weight loss of at least 7.5% of recall body weight (group 3) and 7 patients as for group 3 who had received 7 days of intravenous nutrition in the immediate preoperative period (group 4). Details of the patients are given in tables 3 - 6, pages 92 - 95 which lists age, weight, % weight loss, height, mid arm circumference, triceps skinfold thickness and plasma albumin.

Each patients was allocated a unique study number (up to 62, 4 patients having been withdrawn and the number not reused) regardless of which arm(s) they were entered into.
Table 3. Details of patients in the in vivo study from group 1. '*' indicates participation in the in vivo trial, '#' indicates participation in the in vitro trial

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<th>Weight kg</th>
<th>Height cm</th>
<th>MAC mm</th>
<th>TSF mm</th>
<th>Albumin g/l</th>
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Table 4. Details of patients in the study from group 2. '*' indicates participation in the *in vivo* trial, '#' indicates participation in the *in vitro* trial

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- 93 -
Table 5. Details of patients in the study in group 3. '*' indicates participation in the *in vivo* trial, '#' indicates participation in the *in vitro* trial.

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Table 6. Details of patients in the study in group 4. '*' indicates participation in the *in vivo* trial, '#' indicates participation in the *in vitro* trial

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<td>cm</td>
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<td>mm</td>
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<td>15</td>
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<td>251</td>
<td>4.6</td>
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<tr>
<td>*</td>
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<td>70</td>
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<td>12</td>
<td>161</td>
<td>274</td>
<td>8.8</td>
</tr>
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<td>150</td>
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<td>67</td>
<td>42</td>
<td>19</td>
<td>157</td>
<td>214</td>
<td>9.2</td>
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2. **THE *IN VIVO* STUDY**

Thirty four patients were entered into this arm of the study; 17 with less than 7.5% body weight loss (group 2), 11 with greater than 7.5% body weight loss randomised not to receive preoperative intravenous nutrition (group 3) and 6 with greater than 7.5% body weight loss randomised to receive 7 days of preoperative intravenous nutrition (group 4).

Details of the patients who took part in this arm of the study are given in the tables 4 - 6 on pages 93 - 95. The patients in the *in vivo* study are marked '*'.

The results presented here are analysed firstly to establish that we have observed a typical acute phase response in the patients studied and secondly to look for evidence of the influence of the preoperative nutritional status of the subjects.

Nutritional influence was sought by two methods, firstly by correlating nutritional variables measured in the patients with levels of acute phase proteins post-operatively and secondly by comparison of acute phase protein levels between the 3 groups.

The correlations were performed excluding subjects in group 4 who had received intravenous nutrition since it was felt they may have developed a greater postoperative acute phase reaction than their nutritional assessment would suggest under the influence of nutritional support and this might have introduced distortion.
1. Validation of a normal acute phase reaction.

Figures 3 - 7, pages 98 - 102, show the time course of the acute phase response for each of the five proteins measured, C-reactive protein, alpha₁-antitrypsin, alpha₁-acid glycoprotein, prealbumin and transferrin.

The graphs show that each of these proteins exhibited a typical acute phase reaction, the three conventional acute phase proteins rising and the two transport proteins falling in concentration in response to the trauma of operation. The highest CRP concentration measured was at 48 hours. The highest concentrations of AAT and AAG were measured at 72 hours, and the lowest concentrations of prealbumin and transferrin were seen at 72 hours. Peak and trough levels are therefore taken as the 72 hour value except in the case of CRP when the 48 hour value is taken.
Figure 3. The time course of plasma C-reactive protein concentration (median, Q1-Q3) following operation in all the patients studied.
Figure 4. The time course of plasma alpha\textsubscript{1}-antitrypsin concentration (median, Q1-Q3) following operation in all the patients studied.
Figure 5. The time course of plasma alpha$_1$-acid glycoprotein concentration (median, Q1-Q3) following operation in all the patients studied.
Figure 6. The time course of plasma prealbumin concentration (median, Q1-Q3) following operation in all the patients studied.
Figure 7. The time course of plasma transferrin concentration (median, Q1-Q3) following operation in all the patients studied.
2. Correlation of protein levels with preoperative nutritional indices

Correlations were performed between the following variables.
X axis;
Age (years), height (kg), weight loss (%), height (cm), mid arm circumference (mm), triceps skinfold thickness (mm), plasma albumin (g/l), duration of operation (min) and pathological stage of disease (for key see page 68).
Y axis;
Plasma CRP at 12, 24, 48 and 72 hours.
Plasma AAT at 24, 48, 72 hours
Plasma AAG at 24, 48, 72 hours
Plasma prealbumin at 24, 48, 72 hours
Plasma transferrin at 24, 48, 72 hours

The results of these correlations are given in tables 7 - 9, pages 104 - 106.

Only 11 statistically significant correlations were found in 144 performed, 8 at the 0.05 level and 3 at the 0.01 level. Of these 5 were correlations with the duration of operation. There was no pattern in the other significant correlations noted which are likely to have arisen by chance.
Table 7. Correlations of patient variables with postoperative concentrations of CRP. Values in the table are R, the Spearman rank order correlation coefficient.

* 0.05 > p > 0.01  ** p < 0.01.

CRP 12 = CRP concentration at 12 hours etc.

<table>
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<th>CRP 72</th>
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<td>0.32</td>
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<td>-0.14</td>
<td>-0.02</td>
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<tr>
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<td>0.17</td>
<td>0.12</td>
<td>0.28</td>
</tr>
<tr>
<td>MAC</td>
<td>-0.16</td>
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<td>0.27</td>
</tr>
<tr>
<td>TSF</td>
<td>-0.30</td>
<td>0.11</td>
<td>0.20</td>
<td>0.25</td>
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<tr>
<td>Albumin</td>
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<td>0.12</td>
<td>0.15</td>
<td>-0.01</td>
</tr>
<tr>
<td>Duration op.</td>
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<td>0.02</td>
<td>0.25</td>
<td>0.42*</td>
</tr>
<tr>
<td>Tumour stage</td>
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<td>-0.40*</td>
<td>-0.22</td>
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</table>
Table 8. Correlations of patient variables with postoperative concentrations of alpha$_1$-antitrypsin and alpha$_1$-acid glycoprotein. Values in the table are R the Spearman rank order correlation coefficient.

* 0.05 > p > 0.01  ** p < 0.01.

<table>
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<th>AAT 72</th>
<th>AAG 24</th>
<th>AAG 48</th>
<th>AAG 72</th>
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<td>-0.08</td>
</tr>
<tr>
<td>Wt loss</td>
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<td>0.11</td>
<td>0.09</td>
<td>0.23</td>
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<td>0.13</td>
</tr>
<tr>
<td>Height</td>
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<td>0.04</td>
<td>-0.20</td>
<td>0.14</td>
<td>0.00</td>
<td>-0.12</td>
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<td>-0.21</td>
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<td>0.08</td>
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</table>
Table 9. Correlations of patient variables with postoperative concentrations of prealbumin and transferrin. Values in the table are R, the Spearman rank order correlation coefficient.
* 0.05 > p > 0.01  ** p < 0.01.

<table>
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<th>PRE 48</th>
<th>PRE 72</th>
<th>TRF 24</th>
<th>TRF 48</th>
<th>TRF 72</th>
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<td>-0.49*</td>
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<td>-0.14</td>
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<td>-0.06</td>
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<tr>
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<td>0.05</td>
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<td>-0.16</td>
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<td>0.04</td>
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<tr>
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<td>-0.17</td>
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<td>-0.33</td>
<td>-0.35</td>
<td>-0.14</td>
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</table>
3. Comparison of groups 2, 3 and 4

Table 10, page 108 compares the patients in groups 2, 3 and 4 to establish their comparability using nutritional and other criteria. It is noticeable that there are no statistically significant differences in the nutritional variables between patients in any of the three groups (except weight).

Figures 8 - 12, pages 109 - 113 show the levels of the 5 proteins measured by group of the study. In each of the 5 graphs there are no statistically significant differences between any of the corresponding time points (Mann Whitney U).
Table 10. A comparison of patients from groups 2, 3 and 4 showing nutritional indices stage of disease and duration of operation. Values are median (Q1-Q3). Disease stages are defined on page 68.

<table>
<thead>
<tr>
<th>Group</th>
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<td>72 (68-76)</td>
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<td>Weight (kg)</td>
<td>69 (47-74)</td>
<td>60 (52-65)</td>
<td>53 (42-55)</td>
</tr>
<tr>
<td>Weight loss (%)</td>
<td>0 (0-0)</td>
<td>11 (11-13)</td>
<td>13 (10-16)</td>
</tr>
<tr>
<td>MAC (mm)</td>
<td>271 (221-275)</td>
<td>252 (204-257)</td>
<td>240 (211-257)</td>
</tr>
<tr>
<td>TSF (mm)</td>
<td>9.2 (5.6-10.8)</td>
<td>6.4 (3.1-10.6)</td>
<td>6.8 (3.9-8.5)</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>42 (36-44)</td>
<td>39 (34-41)</td>
<td>37 (35-39)</td>
</tr>
<tr>
<td>Stage of disease</td>
<td>4 (2-4)</td>
<td>4 (3-4)</td>
<td>4 (2-4)</td>
</tr>
<tr>
<td>Duration of op. (min)</td>
<td>255 (213-348)</td>
<td>255 (187-286)</td>
<td>277 (197-337)</td>
</tr>
</tbody>
</table>
Figure 8. The time course of plasma C-reactive protein concentration (median, Q1-Q3) following operation for groups 2, 3 and 4.
Figure 9. The time course of plasma alpha₁-antitrypsin concentration (median, Q1-Q3) following operation for groups 2, 3 and 4.
Figure 10. The time course of alpha_1 acid glycoprotein concentration (median, Q1-Q3) following operation for groups 2, 3 and 4.
Figure 11. The time course of plasma prealbumin concentration (median, Q1-Q3) following operation for groups 2, 3 and 4.
Figure 12. The time course of plasma transferrin concentration (median, Q1-Q3) following operation for groups 2, 3 and 4.
2. THE IN VITRO STUDY

The experiments reported in this section attempt to test the validity of using the liver slice as a model for studying the acute phase reaction in vitro and then seek evidence for the influence of nutrition on the observed responses.

Incubation of the liver samples

Liver incubations were carried out on tissue specimens from 40 patients in total, 19 from group 1, 9 from group 2, 7 from group 3 and 5 from group 4. Details of these patients are to be found marked '#' in tables 3 - 6, pages 92 - 95.

A further 7 patients had biopsies taken but no results are available. Two had their liver samples grossly contaminated by overgrowth of bacteria and the results discarded and in 5 no incubations were carried out due to technical problems at the stage of slicing the liver leading to maceration rather than slicing. These 5 occurred with small biopsy specimens in the early attempts at slicing before a reliable technique was established for fixing the liver specimen to the platter of the McIlwain tissue slicer.

The weight of the liver tissue obtained varied between 0.24 and 2.16 g and was entirely at the discretion of the operating surgeon. No complications occurred in any patient as a result of the biopsy procedure.
The number of incubation dishes which could be set up from each biopsy varied between 2 and 6, depending on the size of the trimmed specimen and the success of the slicing procedure. This was technically the most difficult stage of the preparation of the incubation dishes and was dependent on the degree of fixation of the sample to the platter. Movement of even a minor degree of the specimen coupled to the inherent lack of rigidity of liver parenchyma resulted in considerable variation in the thickness of the liver slices which was readily apparent to the naked eye even at a nominal slicing thickness of 0.4 mm. This is likely to have had considerable implications for the nutrition and oxygenation of the liver parenchymal cells during incubation.

**Intracellular CRP levels**

In order to establish that the CRP appearing in the incubating medium was indeed newly synthesised, the CRP content of fresh liver was measured in 15 samples of liver. The median amount of CRP present was $3.57 \times 10^{-4}$ mcg/mg wet liver ($Q1-Q3$, 2.58 - 4.80; range 1.7 - 7.7).

In addition the intracellular content of 8 samples of liver was measured at the end of the incubation in phase 2 (all incubated with 10 U/ml IL-1) to ensure that the amount of CRP seen in the medium represented all the newly synthesised CRP. The median amount present was $5.26 \times 10^{-4}$ mcg/mg wet liver ($Q1-Q3$, 3.21 - 10.62). Although this is greater than concentration of intracellular CRP measured before incubation, it indicates that most of the
newly synthesised CRP was being released into the medium.

**Effect of interleukin-1 on CRP production**

Every sample of liver obtained was incubated both with and without the presence of interleukin at a concentration of 10 IU/l. The results are shown in figures 13 and 14, pages 117 and 118, showing production of CRP from liver slices in the presence and absence of IL-1 in both the air/CO₂ and O₂/CO₂ phases respectively. In phase 1 the median CRP released into the medium rose from 7.48 to 11.85 x 10⁻⁴ mcg/mg (p = 0.008; Wilcoxon) and in phase 2 from 93.3 to 137 x 10⁻⁴ mcg/mg (p < 0.001; Wilcoxon). The median of the difference of pairs expressed as a percentage of the first of the pair was 84%.

The maximum amount of CRP released into the medium by interleukin-1 stimulated liver slices was 432 x 10⁻⁴ mcg CRP/mg wet liver. Extrapolating by weight this would represent 64.8 mg CRP from a whole 1.5 kg liver. Diluted in 5 litres of serum this would give a concentration of approximately 13 mg/l ignoring the effect of degradation and consumption of CRP. This is only a few fold above basal levels in man suggesting that the liver slices function ineffectively in synthesising CRP compared to intact liver. The levels are however more than 100 fold greater than that which was found in the fresh liver prior to incubation.
Figure 13. The effect of 10 U/l interleukin-1 on CRP production from liver slices in phase 1. \( n = 14; \ p = 0.008 \) (Wilcoxon)
Figure 14. The effect of 10 U/l interleukin-1 on CRP production from liver slices in phase 2. n = 27; p < 0.001 (Wilcoxon)
Dose response of interleukin-1

The dose response relationship of interleukin-1 was investigated in 14 incubations, by varying the dose of IL-1 between 1 and 20 U/ml in steps of 1, 5, 10 and 20 U/ml. Due to either the absence of sufficient IL-1 or low yield of slices, not every increment was used on each occasion. Figure 15, page 120, plots the results obtained.

This initially confusing graph has certain patterns.

1. There is no significant difference between control samples and those stimulated with 1 U/ml IL-1. This dose was abandoned after the first 5 results were available.
2. Five U/ml produced significant stimulation compared to control values.
3. Ten U/ml produced significant stimulation compared to control values.
4. Ten U/ml produced more stimulation than 5 U/ml but this was not statistically significant.
5. There was little difference between 10 U/ml and 20 U/ml (although with 2 very high results), and this was not statistically significant.

The statistical tests are summarised below.
Figure 15. The effect of increasing concentrations of IL-1 in the incubating medium on CRP production of incubated liver slices. The statistical analysis is described in the text. The asterisks represent points at 647 and 681 mcg/mg x 10^{-4} CRP.
All values are median (Q1-Q3) CRP production in mcg/mg wet liver $\times 10^{-4}$, and p value by Wilcoxon.

Control v 1 U/ml;
95 (6.4-100) v 90 (4.5-201) n = 5  ns

Control v 5 U/ml;
103 (9.3-95.5) v 161 (16.9-132) n = 12  p = 0.012

Control v 10 U/ml;
103 (9.3-95.5) v 192 (20.4-156) n = 12  p < 0.01

5 U/ml v 10 U/ml;
161 (16.9-132) v 192 (20.4-156) n = 12  p = 0.45

10 U/ml v 20 U/ml;
160 (90.6-194) v 145 (77.5-200) n = 11  p = 0.85

**Effect of varying the oxygenation of the tissue slices**

The liver slices were initially incubated in an atmosphere of 95% air/5% CO$_2$ (phase 1). This was later changed to an atmosphere of 95% O$_2$/5% CO$_2$ (phase 2) in order to improve the preservation of the liver cells. The change of gas phase had a considerable effect on the amounts of CRP produced. Figure 16, page 122 shows the distribution of CRP production in samples incubated for 24 hours in the presence of 10 U/ml interleukin-1 for phase 1 and phase 2. The improved oxygenation had a clear effect on CRP production, increasing median CRP production by approximately 13 fold, from 10.8 to $137 \times 10^{-4}$ mcg/mg wet liver ($p < 0.001$; Mann Whitney U). This must be taken into account when other aspects are analysed since phase 1 and phase 2 are not therefore comparable.
Figure 16. The effect of increasing the tissue oxygenation on the CRP production of liver slices incubated for 24 hours (phase 1 (n = 14) v phase 2 (n = 27)). p < 0.001 (Mann Whitney)
Time course of CRP production

The initial incubations were carried out in an atmosphere of 95% air, 5% CO$_2$ and for 24 hours. In 2 sets of experiments this period was extended to 96 hours, and measurements were made of the CRP concentration in the medium every 24 hours. At each of the specified time intervals 0.5 ml of medium was removed and replaced with 0.5 ml of fresh DMEM with or without FCS but without further interleukin-1 thus slightly altering the composition, diluting products of metabolism and IL-1 and renewing substrate supply.

The amount of CRP present in the medium at each time interval is shown in figures 17 and 18 (pages 125 and 126). Each graph represents several incubations from a single patient with varying composition of the medium. It can be seen that CRP production continued to rise in a near linear fashion for 96 hours. This suggests that the hepatocytes in the culture remained viable throughout this period. This is at variance with the histological evidence detailed below.

The early time course of CRP production was also studied by measuring both intracellular CRP and CRP released into the medium at intervals of 2, 4, 6 and 24 hour of incubation using the last patient from phase 1. The results are shown in figures 19 and 20, pages 127 and 128, representing the control and IL-1 stimulated liver slices. It can be seen that there is a lag phase with little increase in the total amount of CRP present within first 4 - 6 hours. This is in keeping with the similar lag
phase known to occur in the \textit{in vivo} acute phase reaction. These graphs also show that the intracellular CRP is not substantially greater at 24 hours than at the beginning of the incubation.
Figure 17. The time course of CRP production of eight incubated liver slices from patient no 9. Although there are differences in the composition of the medium CRP synthesis continues for 96 hours in all the slices incubated.
Figure 18. The time course of CRP production of seven incubated liver slices from patient no 10.
Figure 19. The early time course of CRP production in parallel incubations from patient no 17 without exogenous IL-1. \( C_T \) = total CRP; \( C_M \) = CRP released into the incubating medium; \( C_I \) = intracellular CRP.
Figure 20. The early time course of CRP production of parallel incubations from patient no 17 in the presence of 10 U/l IL-1. \( IL_T \) = total CRP; \( IL_M \) = CRP released into the incubating medium; \( IL_I \) = intracellular CRP.
Effect of foetal calf serum on CRP production

The effect of the presence in the medium of foetal calf serum was investigated in 20 subjects, 6 from phase 1 and 14 from phase 2, who had paired incubations set up with and without foetal calf serum. Neither of the pair contained interleukin-1.

There was no significant difference between the pairs when analysed altogether or separating phase 1 and phase 2 incubations. These results are shown graphically in figure 21, page 130.

From 13 of this group of 20 subjects incubations were also set up with 10 IU/l interleukin-1 both with and without FCS, thus having four parallel sets of incubations (with and without IL-1, with and without FCS) under otherwise identical conditions. The results of these are shown in figure 22, page 131.

The statistics are as follows. All values are median (Q1-Q3) CRP production in mcg/mg wet liver x 10^-4. Groups compared by Wilcoxon.

FCS+/IL1- v FCS-/IL1-  95 (14-115) v 101 (21-190)  ns
FCS-/IL1+ v FCS-/IL1-  127 (18-159) v 101 (21-190)  ns
FCS+/IL1+ v FCS+/IL1-  192 (25-258) v 95 (14-115)  p = 0.001
FCS+/IL1+ v FCS-/IL1+  192 (25-258) v 127 (18-159)  p < 0.01
FCS+/IL1+ v FCS-/IL1-  192 (25-258) v 101 (21-190)  p < 0.05
Figure 21. The effect of foetal calf serum on the production of CRP in the absence of interleukin-1. n.s.
Figure 22. The effect of foetal calf serum both with and without interleukin-1 in parallel incubations. The statistical analysis is described in the text.
The results shown in the preceding graphs may be summarised as follows;
1. FCS had no significant effect on unstimulated liver
2. IL-1 had no significant effect in the absence of FCS
3. FCS together with IL-1 stimulated CRP production compared with only one of these or neither.

**Cell Viability**

Cell viability and preservation during the incubation period was assessed by 3 methods.

1. Trypan blue staining.

The results obtained by tissue digestion and trypan blue staining were uniformly unhelpful. Four sets of liver samples were examined by this method. Despite carefully following the method recommended by the manufacturer of the trypan blue, all samples showed total fragmentation of all the cells with no intact cells seen. Since this did not accord with the biochemical evidence of continuing CRP production by these cells this method was abandoned early in the course of the experiments, in the belief (probably erroneous) that the fragmentation was being caused by the trypsin digestion and centrifugation processes rather than the conditions of handling and incubation of the liver tissue.

2. Comparison of intracellular enzyme activity of fresh liver tissue and the liver slices at the end of the incubation period.
Ten pairs of enzyme levels were measured, all from the $O_2/CO_2$ phase of the study. Table 11, page 134 gives the levels of phosphofructokinase, fructose 1,6-diphosphatase and hexokinase before and after incubation, and table 12, page 135 expresses the post incubation levels as a percentage of the pre incubation levels. The post incubation samples were all incubated in the presence of interleukin at a concentration of 10 IU/l.
Table 11. Intracellular glycolytic enzyme levels in liver tissue before and after incubation. Values are mcmol product/min/g wet liver. PFK = phosphofructokinase, FDP = fructose 1,6-diphosphatase.

<table>
<thead>
<tr>
<th>Study number</th>
<th>PFK</th>
<th></th>
<th>FDP</th>
<th></th>
<th>Hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre</td>
<td>post</td>
<td>pre</td>
<td>post</td>
<td>pre</td>
</tr>
<tr>
<td>38</td>
<td>5.33</td>
<td>2.51</td>
<td>4.84</td>
<td>2.59</td>
<td>1.82</td>
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<tr>
<td>39</td>
<td>4.46</td>
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<td>3.92</td>
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<td>0.64</td>
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<tr>
<td>44</td>
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<td>4.74</td>
<td>4.36</td>
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<tr>
<td>45</td>
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<td>4.37</td>
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<tr>
<td>51</td>
<td>4.74</td>
<td>3.07</td>
<td>3.32</td>
<td>2.99</td>
<td>0.52</td>
</tr>
<tr>
<td>53</td>
<td>4.46</td>
<td>3.57</td>
<td>3.40</td>
<td>3.07</td>
<td>1.16</td>
</tr>
<tr>
<td>54</td>
<td>5.75</td>
<td>1.67</td>
<td>5.13</td>
<td>1.67</td>
<td>1.20</td>
</tr>
<tr>
<td>55</td>
<td>2.43</td>
<td>1.78</td>
<td>2.43</td>
<td>1.60</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Table 12. The post incubation glycolytic enzyme levels expressed as a percentage of pre-incubation levels. PFK = phosphofructokinase, FDP = fructose 1,6-diphosphatase.

<table>
<thead>
<tr>
<th>Study no</th>
<th>PFK</th>
<th>FDP</th>
<th>Hexokinase</th>
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<tr>
<td>38</td>
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<td>36</td>
</tr>
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<td>32</td>
<td>82</td>
<td>91</td>
</tr>
<tr>
<td>43</td>
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<td>21</td>
<td>11</td>
</tr>
<tr>
<td>44</td>
<td>82</td>
<td>92</td>
<td>100</td>
</tr>
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<td>45</td>
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<td>90</td>
<td>68</td>
</tr>
<tr>
<td>54</td>
<td>29</td>
<td>33</td>
<td>68</td>
</tr>
<tr>
<td>55</td>
<td>73</td>
<td>66</td>
<td>90</td>
</tr>
<tr>
<td>Median</td>
<td>41</td>
<td>74</td>
<td>75</td>
</tr>
<tr>
<td>Q1-Q3</td>
<td>28-77</td>
<td>27-90</td>
<td>49-90</td>
</tr>
</tbody>
</table>
3. Histology of the liver slices

Histological examination was performed on representative samples of liver from both the air/CO₂ and O₂/CO₂ phases and after various time periods of incubation. Samples of liver were also examined after slicing and before incubation to examine the effects of the slicing procedure on the hepatic parenchymal cells.

Prior to incubation the cells were histologically normal apart from showing some slight degree of fatty vacuolation commonly seen following a period on intravenous nutrition [Tulikoura and Huikuri, 1982]. Trauma to the cells was confined to the extreme periphery of the slices.

In the air/CO₂ phase of incubations there was rapid and generalised deterioration in the histological appearances of the cellular structure. The progression of changes is shown in plates 1 - 5, pages 228 - 230, from patient number 17. The histological appearances were reported as follows;

1. Fresh specimen. "Normal liver in which most cells contain stainable glycogen." (plate 1)
2. Two hours. "Although cytologically the liver cells appear normal there is diminished glycogen content." (plate 2)
3. Four hours. "There is now a definite increase in eosinophilia in periportal and midzonal (Acinar zones 1 and 2) hepatocytes associated with nuclear chromatin condensation indicative of early pyknosis. In contrast the centrilobular (zone 3) cells are pale with normal nuclear..."
morphology. In some areas the glycogen content is slightly greater in zone 3 than in zones 1 and 2, but this is not consistent." (plate 3)

4. Six hours. "Most cells in zones 1 and 2 show eosinophilia and nuclear pyknosis. Zone 3 cells remain pale and have normal nuclei." (plate 4)

5. Twenty four hours. "The liver is totally necrotic. Some pyknotic nuclei persist mainly in zone 3, but elsewhere the cells are eosinophilic 'ghosts' showing gradual disappearance of nuclei by karyolysis." (plate 5)

Similarly liver from study number 15 was reported as follows;

Fresh specimen. "The liver biopsy shows non specific changes of mild fatty change and a focal mild acute inflammatory infiltrate. The architecture appears normal."

Twenty four hours. "The liver cells are completely necrotic. There are no viable hepatocytes."

Six samples in all were examined from phase 1 and all showed complete necrosis at 24 hours.

In phase 2 the liver samples were considerably better preserved though there was a great deal of variation from slight to almost complete necrosis by 24 hours. Examples are described below. Study number 28 (plates 6 - 10, pages 230 - 232) is reported as follows;

1. Fresh specimen. "Within normal limits". (plate 6)

2. Twelve hours. "About 20% of hepatocytes are necrotic. The necrosis is confluent and mainly involves zone 3 cells but in at least one place necrotic cells surround a portal
tract." (plate 7)

3. Eighteen hours. "20 - 30% necrosis in this sample principally involving zone 1." (plate 8)

4. Twenty four hours. "About 10% necrosis seen around the biopsy margins and in periportal zones." (plates 9 and 10).

The fact that there was a greater degree of necrosis recorded at 18 hours than at 24 hours indicates the variability of preservation. This is probably accounted for by the variability of trauma and slice thickness produced at the slicing stage.

By contrast study number 25 is reported;

1. Fresh specimen. "Normal apart from some crush artefact around the margins."

2. Eight hours. "About 80% degenerate - all zones."

3. Twenty four hours. "Totally necrotic."

Fifteen samples from 8 patients were examined in total from phase 2. The median fraction of necrotic cells after 24 hours incubation was 55%.

Reproducibility

Twenty six liver samples were incubated in pairs under identical conditions of medium and oxygenation in order to test the reproducibility of the incubation process. The CRP production from the pairs has been plotted in figure 23, page 139 with the lesser amount on the X-axis and the greater value on the Y-axis. The line of best fit has a slope of 1.27, Rs = 0.968, p < 0.001 (Spearman's rank order correlation coefficient). The
Figure 23. Correlation of values of CRP production from pairs of liver slices incubations under identical conditions of medium and gas phase. The lesser values are on the X-axis. Rs = 0.968, p < 0.001; Spearman.
median of the differences between pairs expressed as a percentage of the first of the pair was 29%, which is considerably less than the differences produced by either the change in the gas phase of the incubation, or the addition of interleukin-1, which were approximately 1300% and 84% respectively.

Influence of nutrition on CRP production

Due to the change in methodology in the middle of the study altering the gas phase of the incubation conditions with its pronounced effect on CRP production, some difficulty has been encountered in this analysis. The already small numbers in each arm are therefore divided into two non comparable groups. The numbers are:

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19 (7 in phase 1 and 12 in phase 2)</td>
</tr>
<tr>
<td>2</td>
<td>10 (2 in phase 1 and 8 in phase 2)</td>
</tr>
<tr>
<td>3</td>
<td>7 (3 in phase 1 and 4 in phase 2)</td>
</tr>
<tr>
<td>4</td>
<td>5 (2 in phase 1 and 3 in phase 2)</td>
</tr>
</tbody>
</table>

Comparisons between groups with such small numbers would not be expected to show significant differences and does not (data not shown). Therefore an analysis of the results has been carried out by correlation between the nutritional indices and CRP production in interleukin-1 stimulated liver. This has been performed separately for phase 1 and phase 2. The correlations are listed in table 13, page 141. Only 2 out of 20 correlations have achieved statistical significance at the 0.05 level. This may have arisen by chance. Interestingly the correlation with weight had a negative value.
Table 13. Correlations of nutritionally related variables with CRP production from interleukin-1 stimulated liver in vitro. Values are Rs (Spearman rank order correlation coefficient). *p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.42</td>
<td>-0.03</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.70*</td>
<td>-0.20</td>
</tr>
<tr>
<td>Height</td>
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<td>0.04</td>
</tr>
<tr>
<td>MAC</td>
<td>-0.51</td>
<td>-0.15</td>
</tr>
<tr>
<td>TSF</td>
<td>-0.36</td>
<td>0.08</td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.67*</td>
<td>-0.05</td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0.24</td>
<td>-0.17</td>
</tr>
</tbody>
</table>
DISCUSSION

Hauf his soul a Scot maun use
Indulgin' in illusions,
And half in gettin' rid o' them
And comin' to conclusions

Hugh Macdiarmid

from A Drunk Man Looks at the Thistle
1. DESIGN OF THE STUDY

The assumptions on which this study was based were that;

i) malnutrition causes disordered organ function and delayed healing leading to an increased incidence of morbidity and mortality in patients undergoing operations

ii) the acute phase reaction and in particular the plasma protein changes seen after trauma and sepsis appear to be a significant part of the process of repair and resolution of inflammation and infection.

The hypothesis was therefore made that a reduction in the magnitude of the acute phase reaction might be expected in malnourished patients and that this might contribute to the increased incidence of complications and mortality. The aim of this study was to test the first part of this hypothesis.

The simplest way to do this was to measure acute phase proteins in the plasma of patients undergoing operation and to relate the plasma concentrations reached to the nutritional status of the patients.

However, since the magnitude of the operation in itself was likely to be a major determinant of the magnitude of the acute phase reaction it was necessary to standardise the degree of surgical trauma as much as possible. This would have been relatively easy by choosing an operation such as herniorrhaphy or cholecystectomy where the degree of dissection should be quite similar in
most patients. However no two surgical operations are exactly the same and more so is this true when multiple operators are involved.

Operations such as herniorrhaphy and cholecystectomy are only rarely knowingly carried out on patients with significant degrees of malnutrition and it was therefore not appropriate to choose such operations. In order to find an elective operation which is commonly carried out in the presence of malnutrition it was necessary to look at the upper gastrointestinal tract and malignant disease in particular.

Gastro-oesophageal cancer is a particular interest of the University Department of Surgery at the Leeds General Infirmary and a large number of operations for this condition are carried out each year. In the department I had already set up a clinical trial in which patients with upper gastrointestinal cancer who were deemed to be malnourished as defined by the loss of 7.5% of recall body weight were randomised to receive 7 days of intravenous nutrition or to act as controls. I therefore decided to use these patients as subjects for the study. In addition a group of patients undergoing surgery for the same condition but without weight loss was also recruited.

Gastro-oesophageal carcinoma is commonly a cause of significant malnutrition by the nature of the disease, and yet surgery must proceed before this malnutrition can be fully or even partially in some cases corrected. Unfortunately operations for gastro-oesophageal carcinoma represent a quite wide range of degrees of surgical
trauma. Even when a rigid protocol of tumour and lymph node excision is followed the length and nature of the dissection depends on the experience and skill of the operator, the build of the patient, the presence of adhesions from previous surgery, the fixity of the primary tumour, the presence of enlarged lymph nodes, either reactive or due to secondary deposits, amongst other factors. For these reasons, ensuring a standard degree of surgical trauma was not a realistic expectation.

An attempt to analyse results within groups stratified to allow for these variables would lead to impossibly small numbers for any realistic hope of achieving statistical significance.

Another factor which masks the role of nutrition is that component of the acute phase reaction produced by pre-existing or early developing infection in patients after major upper gastrointestinal tract surgery, who have a higher incidence of chest and anastomotic infection problems than in lower gastrointestinal tract surgery for instance. There is no method of making an allowance for this when comparing plasma protein concentrations.

For these reasons, I did not feel that the measurement of plasma proteins post operatively was likely to be sufficient in itself to determine whether nutritional status was exerting an influence on the acute phase reaction unless nutrition was the dominant determinant of the magnitude of the acute phase plasma protein response. Nevertheless since I thought that this
problem had not been previously addressed I decided to proceed with this part of the study.

The plasma protein study was designed initially with the expectation that only CRP would be measured, this being the archetypal acute phase protein, easily measured and fast reacting. The intervals at which blood samples were obtained namely at 12, 24, 36, 48 and 72 hours post-operatively was determined by this expectation, in order to observe the expected rise, peak and initial fall of CRP plasma concentration. For many patients having their operation in the afternoon the 12 and 36 hour blood samples fell due in the middle of the night. These samples were only taken from patients who had indwelling vascular access which allowed blood sampling without disturbing the patient. This accounts for the reduced number of samples from these times.

After the study was commenced the opportunity to measure further plasma proteins became available. The proteins measured were one rapidly reacting acute phase protein (CRP), two more slowly reacting proteins (AAT and AAG) and as a contrast two 'negative acute phase reactants', the transport proteins transferrin and prealbumin. CRP was expected to peak at 48 hours post-operatively and the other two reactants at 72 hours or later. Had it been anticipated that I would measure all five proteins from the outset a further one or two days blood samples would have been taken to be sure of reaching the peak plasma concentration of the slower responding reactants.
For the reasons outlined above, a second arm to the study was required which could assess the ability of the patient to mount an acute phase response independent of the operative and postoperative influences referred to, and yet would reflect the preoperative condition of the patient.

The acute phase plasma protein response has two stages, firstly the production of cytokines from macrophages at the site of inflammation and injury, and secondly the stimulation of the liver by these cytokines to synthesise acute phase proteins. At least two groups of workers have shown that malnutrition produces an attenuated response of macrophages to stimuli of IL-1 synthesis and release (see chapter 3 of the introduction). It therefore seemed obvious to study the second stage of the response by looking at the effect on liver of a standardised stimulus in vitro under controlled laboratory conditions.

A liver biopsy taken at the beginning of operation just after the abdomen is opened afforded the ideal material for study. The amount of liver required could only be obtainable by open liver biopsy. Although this might be regarded as a hazardous procedure for patients to undergo for purely research purposes, experience has shown that this is not so in practice. There is a long tradition in the Leeds General Infirmary of research carried on liver samples obtained this way without mishap. The principle hazards of the procedure are the risks of
haemorrhage or bile leak from the cut surface of the liver. Provided the biopsy is carried out properly the risk is low and if carried out at the beginning of a long operative procedure any such problem should be noticed and dealt with by standard means before the abdomen is closed.

The only incident related to the biopsy in this series was in a patient who developed liver necrosis and abscess formation requiring relaparotomy. At re-operation the liver necrosis although in the region of the biopsy site was of such a size, greater than a hundred times the volume of the biopsy, that I was confident that the biopsy was not the cause. This patient had had a radical lymphadenectomy from around the hepatic artery carried high into the porta hepatis suggesting that the cause of the liver necrosis was ischaemia due to vascular damage during the dissection and this was also the opinion of the operating surgeon. Thus I am satisfied that no harm occurred to any patient due to the experimental procedure.

I then had to decide in what way to process the liver sample so obtained. The first possibility considered was to attempt to culture the cells. This was fairly easy to dismiss since culture of normal human hepatocytes had not been previously achieved. In any case the establishment of stable cell cultures would tend to remove the characteristics which reflect the nutritional status of the patient from which the sample was obtained.

The next question was whether to maintain the architecture of the liver intact by using thin slices of liver or whether to attempt to produce a single cell
suspension or an acellular homogenate. Since IL-1 was thought to produce its action via a cell surface receptor and to require an intact system of transcription of mRNA the acellular homogenate was rejected. At the time of designing the experimental method it was still not certain whether IL-1 was acting directly on hepatocytes or whether a second messenger produced in the Kupffer cells was required, and if so whether an intact liver architecture would be required. For this reason it was decided to use liver slices as the model.

There was in the department a purpose built machine for this purpose the McIlwain tissue slicer [McIlwain and Buddle, 1953] and this was used to produce the liver slices. In practice it proved not to be an ideal tool. Difficulty in fixing the specimen to the platter was experienced and a number of methods of fixing the specimen were tried including the use of agar, wetting the platter and finally allowing the liver to dry slightly on a piece of filter paper which was then attached to the platter with metal clips. Time on the platter is 'warm ischaemia time' being out of the iced saline and variability here could only affect the preservation of the cells quite considerably, both from the effect of temperature and desiccation. The McIlwain slicer is more suited to the slicing of tissue that is less sensitive to warm ischaemia and is firmer in consistency so that it may be sliced more consistently. These difficulties have been recognised before and an improved slicer has been described
[Krumdieck, 1980]. Using this device human liver slices have been produced with a consistent measured thickness and incubated with virtually complete histological preservation at 20 hours [Smith et al, 1985]. Attempts to find a manufacturer for this device proved fruitless despite contacting the original designer directly.

The next problem to be addressed was the duration of incubation. CRP is not stored in hepatocytes but synthesised de novo which involves a lag phase of at least 6 hours. It was not known what the rate of protein synthesis would be in this model and I did not know how long it would take to synthesise sufficient CRP to be detectable by the assay which had a sensitivity of 0.1 mcg/l. The duration of incubation required was important in deciding the conditions under which to maintain the liver slice. The period chosen was 24 hours initially but in early experiments I extended this to 96 hours in 4 patients (nos 8 - 11). When the results of histological examination were obtained showing the destruction of the liver architecture and phase 2 was started the period was restricted to 24 hours.

The medium chosen was a standard commercially available one the use of which for maintaining hepatocytes is well documented [Baumann et al, 1984]. Dulbecco's modified Eagle medium (DMEM) uses bicarbonate as a buffering system which dictated the need for an atmosphere containing 5% CO₂ to maintain the pH of the medium at 7.4.

I elected on the grounds of simplicity to use a 95% air/5% CO₂ atmosphere for the initial incubations which
could be provided automatically in an incubator which was in our laboratory. Evidence that this would maintain a significant proportion of hepatocytes within liver slices for a period of at least 24 hours was found in previous work from Cambridge using rat liver, where necrosis occurred between 24 and 48 hours [Campbell and Hales, 1971]. This in retrospect was an error which had significant consequences for the success of the project.

A controversial area was the need for a natural serum in the incubating medium. Although necessary for the prolonged culture of some tissues due to the requirement for as yet unidentified factors, the unknown contents of sera such as foetal calf serum (FCS) has led to a desire to dispense with these wherever possible in order to have only precisely defined elements in the medium [Taylor, 1974]. Three questions arose with regard to my experiment. Firstly did the IL-1 require to be cleaved to smaller fragments before it would become active, as had been suggested for its proteolysis inducing action [Clowes et al, 1986]. Secondly, did the FCS contain any cytokines or substances which might induce cytokine production in Kupffer cells? Although the FCS is tested for endotoxin many other potent stimulators of the acute phase reaction may have been present. Thirdly, did the FCS contain any IL-1 inhibitors? These had been detected in human urine [Liao et al, 1984; Liao et al, 1985] and presumably must be present in plasma too. Since it was not clear what the effect of FCS would be in my system, I elected to carry
out parallel incubations both with and without the addition of 5% FCS.

The dose of IL-1 to be added was decided from previous work on hepatocyte cultures. Since the release of some IL-1 from the Kupffer cells within the slices could not be excluded or allowed for then a supramaximal stimulus was required in order to standardise the response even if this was not a reflection of what would occur in vivo in the postoperative state. Work with cultured mouse hepatocytes and recombinant human IL-1 had suggested [Ramadori et al, 1985] that maximal stimulation was obtained by a dose of approximately 10 U/ml IL-1 and so this concentration of IL-1 was adopted. The use of an even higher dose to ensure that the stimulus was supramaximal was prevented by the price of recombinant IL-1. Every incubation dish required £15 of IL-1 at 1987 prices and up to 6 dishes were incubated per patient. In a small number of incubations a dose response curve was produced by using concentrations varying between 1 and 20 U/ml to check the validity of the assumption.

Testing of the maintenance of cell viability was initially attempted by the method of trypan blue staining. This works on the principle that after separation by trypsin digestion only intact viable cells will take up the dye trypan blue. When the stained cells were examined under the microscope only fragmented cells were seen, and this was erroneously thought to be due to destruction at the trypsinisation or centrifugation stages of the procedure. I was encouraged in this view by the initial
assays of CRP given off by the incubated liver slices which continued to increase in a linear fashion for 96 hours (see figures 17 and 18, pages 124 and 125).

With presumed failure of the method of trypan blue staining, histological examination was used to look at the liver slices. This was not used initially since it was thought not sensitive enough to detect the subtle changes in morphology that might affect function. Unfortunately histological examination showed that frank necrosis of the hepatocytes had taken place well within 24 hours. I felt that with such gross necrosis it would be difficult to extrapolate the activity of these slices to events in vivo and therefore a revision of the method was required after 14 sets of incubations had been carried out.

The technique of organ culture has been applied to several types of tissue with variable degrees of success related principally to the degree of metabolic activity of the tissue concerned, with metabolically less active tissue such as cartilage, bone and prostate having greater success in maintaining stable tissue specimens than more demanding tissues such as muscle, kidney and particularly liver which has been the most difficult tissue to maintain for extended periods of time. In order to improve the preservation of my liver samples previously described methods which had been successful in maintaining histologically normal mammalian liver for periods of up to six days were adopted. The principle improvement was to increase the oxygenation of the hepatocytes, thought to be
the most critical factor in maintaining cellular integrity, thus achieving better preservation of the liver slices.

The liver slices were therefore suspended at the surface of the liquid by mounting them on a stainless steel mesh. By bending a small strip of this mesh a flat platform was created just under the surface of the medium maintaining the explants at the gas/liquid interface. Similar platforms were described by Trowell in the first definitive description of organ culture methods [Trowell, 1958]. The alternative to suspending the cells at the gas/liquid interface was to maintain the medium and tissue slice in continuous motion to ensure uniform gas distribution throughout the medium. Such systems have been widely described [Balls and Clothier, 1983; Smith et al, 1985]. In addition the atmosphere was altered to 95%O₂/5%CO₂ by placing the incubation dishes, mounted on a specially made perspex holder, in a standard McIntosh Fieldes anaerobic jar and filling the jar with a premixed gas of the appropriate composition. Subsequent histological examination showed marked improvement in the morphology of the liver slices at the end of the incubation period, though this remained inconsistent and imperfect (see pages 136 et seq) and inferior to previously described work.

In order to demonstrate the degree of functional preservation of the liver the activity of 3 enzymes important in the glycolytic pathway were determined before and after incubation. The enzymes were chosen from their
central role in cellular metabolism and because there was experience within the department of their measurement [King et al, 1981]. Table 11, page 134 shows the absolute values obtained and table 12, page 135 expresses the post incubation activities as a percentage of the pre incubation levels. Interestingly phosphofructokinase (PFK) was less well preserved than the other two which maintained about 75% of preincubation activity, though again there is considerable variation between experiments varying between 3 and 82% for PFK and 36 and 100% for hexokinase which was the best preserved. The variability in biochemical preservation thus reflected the variation in architectural preservation seen on histological examination. Unfortunately a slice could not be both examined histologically and have enzyme assays carried out due to the small size of the slices approaching the limit of sensitivity of the assay and so no direct comparison was possible.

From the above discussion it will be seen that a major difficulty in carrying out this study was the need to alter the experimental method after a number of patients were recruited. I started the study before knowing how best to carry it out. Once started I was then reluctant to change the incubation methods since this would destroy the comparability of the experiments before and after any change was made, and this was only done once it was clear that the tissue necrosis was so severe as to bring into question the validity of the method. This
effectively made the first phase of the work a pilot study
the results of which were not strictly comparable with the
second phase.

Had a limitless supply of patients suitable for
recruitment been available this would not have been
important. However, the numbers of patients who could be
recruited in the two year period which it was anticipated
would be available for this study were not high. In
practice many suitable patients could not be recruited,
mostly due to unwillingness to give permission for liver
biopsy, but also because of technical failures, especially
of the slicing mechanism, and running out of reagent (the
interleukin-1 was delivered on a regular weekly basis on
account of its limited shelf life which did not always
match the supply of patients).

The power of the statistical analysis which could be
carried out to address the original question of the
influence of nutrition was thereby compromised by the
small numbers in the two phases of the study.
2. RESULTS OF THE IN VIVO STUDY

Before examining the effect of nutrition on the magnitude of the acute phase plasma protein response I had to establish firstly that a normal acute phase reaction was being observed and secondly that the study group of patients was malnourished.

The first of these was dealt with by comparing the graphs on pages 98 - 102 with previously described graphs of the time course of acute phase plasma protein concentration following similar types of surgery. The rise in median CRP from 0.6 mg/l, the lower limit of the assay, to 144 mg/l is compared in table 14, page 158, with peak levels seen in previously documented studies where the acute phase plasma protein response had been followed postoperatively. The measured levels vary considerably, but a clear acute phase reaction with levels in the expected range was seen in this series. The occurrence in a few patients of CRP concentrations above the normal range in the preoperative sample did not lead to higher peaks of CRP. This has been noted by previous experimenters [Colley et al, 1983].

The quantification of malnutrition is an imprecise science for reasons that are outlined in section 3 of the introduction of this thesis. The choice of weight loss as the single criterion to select the malnourished population depends on the conclusion that failure to maintain body weight is the clearest indication of inadequate recent nutrition. Although weight loss will be affected by the
Table 14. Peak acute phase protein levels in this study compared to previously documented series. All values are mg/l.

<table>
<thead>
<tr>
<th>Operation</th>
<th>AAT</th>
<th>AAG</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastectomy</td>
<td></td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>Cholecystectomy</td>
<td>1450</td>
<td>130</td>
<td>b</td>
</tr>
<tr>
<td>Herniorrhaphy</td>
<td>1300</td>
<td>115</td>
<td>b</td>
</tr>
<tr>
<td>Various</td>
<td>5000</td>
<td>1600</td>
<td>165</td>
</tr>
<tr>
<td>Various</td>
<td>c200</td>
<td></td>
<td>d</td>
</tr>
<tr>
<td>Gastrectomy</td>
<td>1570</td>
<td></td>
<td>e</td>
</tr>
<tr>
<td>G-I resection</td>
<td>3900</td>
<td>1650</td>
<td>210</td>
</tr>
<tr>
<td>This study</td>
<td>3800</td>
<td>1190</td>
<td>144</td>
</tr>
</tbody>
</table>

complex relationship between weight loss and malignancy (see page 47), other nutritional markers will be also affected by factors other than nutrition. The figure of 7.5% is an arbitrary one but represents approximately one stone in a man of average build which would be regarded by most people as a significant loss of weight in a three month period.

However in the patients studied here this amount of weight loss has not been accompanied by significant differences in other accepted nutritional indices such as mid arm circumference, triceps skinfold thickness or the plasma concentrations of albumin and transferrin. Unexpectedly there was no significant statistical difference in actual weight between the group with weight loss and those without, which suggests that the malnourished group may possibly have started from a higher basal weight than the no weight loss group. Another possibility is that patients recall of actual weight and weight loss is inaccurate and thus unreliable as a method of assessing nutritional status. Nevertheless all the nutritional parameters did trend in the direction expected in the weight loss group and p values were close to the 0.05 level and would probably have achieved significance with slightly greater numbers in each group.

When the plasma concentrations of the acute phase proteins were compared in the groups as defined there was no significant difference in the peak levels of CRP, AAT or AAG and no discernible trend (figures 8 - 10, pages 109 - 111).
In order to assess whether this result had arisen because of an erroneous choice of a marker for malnutrition, ie weight loss, I decided to seek correlations between the measured nutritional variables and the levels of acute phase plasma proteins in the post operative period.

This should show any genuine association and avoided the need for comparisons of groups selected on arbitrary parameters of nutritional variables such as weight, weight loss, anthropomorphic measurements or plasma albumin. Correlations were also sought with duration of operation as a measure of the operative trauma and stage of disease since there is evidence that acute phase response may be attenuated by malignant disease [Herman et al, 1984]. The results are shown in tables 7 and 8 on pages 104 and 105. Although some significant correlations do appear this would be expected even on a random basis when this number of correlations are performed and no consistent pattern emerges. One unexpected trend was the inverse relationship between both AAT and AAG concentrations and the duration of operation.

Similarly when the transport protein profiles were correlated in the same manner (table 9, page 106) no consistent pattern emerged.

Since the experimental work on this study was completed I have found two publications on the effect of nutritional status on the acute phase response to major surgery, one from the group in Milan who in 1984 announced
that no study had been carried out [Dionigi et al, 1984] and published shortly afterwards [Dominioni et al, 1984] and a second from Glasgow published in 1989 [Cruikshank et al, 1989]. Both purport to show a reduced acute phase response in malnourished patients but in both the effect is slight. The Glasgow group selected patients on the basis of being underweight, i.e. less than 80% of ideal body weight, without specifying actual weight loss. They then measured CRP, AAT and AAG in their patients after a variety of abdominal procedures. AAT and AAG showed no difference but the peak CRP level was reduced by a significant level p < 0.02. This is open to the criticism that these subjects were thin normals rather than malnourished. Also since weight reached a significant correlation with CRP (but not with AAG or AAT) in this study the results may be consistent ie lean normal subjects may produce a lower peak of CRP concentration than heavier subjects whether or not they are malnourished.

The Milan group found a weak relationship with only AAG (but not AAT or CRP) showing a significant reduction in peak concentration compared to normal subjects after gastrectomy when patients were selected on the basis of serum albumin < 32 mg/l. No such relationship was seen in the present study.

The reasons why no relationship has been demonstrated between nutritional status and the magnitude of the acute phase response need to be examined.

The first possibility is that there is no
relationship between malnutrition and the acute phase response, and that except in very advanced cachexia the acute phase response is a favoured pathway of hepatic protein synthesis at the expense of albumin and transport proteins on account of the biological importance of acute phase proteins in recovery from trauma and infection. This suggestion has been made previously [Dionigi et al, 1984].

A second possibility is that a relationship does exist but that it is weak and insufficient numbers of patients have been recruited to demonstrate it. This cannot be tested without doing the necessary experiment with far greater numbers of patients.

It is also possible that a relationship exists which is being masked by other factors influencing the acute phase response, such as degree of surgical trauma and tumour load which have not been adequately controlled for. As I have discussed above a reproducible degree of surgical trauma is not a real possibility except in very minor procedures.

Finally it may be that the patients in this study are not malnourished, that 7.5% loss of body weight does not represent a sufficient loss of body tissue to justify the term malnourished, and that an effect in the small numbers of genuinely malnourished with greater degrees of weight loss was masked by the patients with lesser degrees of weight loss in the malnourished group. Unfortunately there are insufficient numbers with large (\(>15\%\)) degrees of weight loss to make a meaningful statistical analysis.
3. RESULTS OF THE IN VITRO STUDY

The first objective with the liver slice incubations was to establish that the liver samples were capable of synthesising CRP, and that they were responsive to the exogenous IL-1. This required firstly the assay of intracellular CRP in fresh liver to ensure that CRP released into the medium was not simply the release of stored CRP and secondly the measurement of CRP production from liver slices incubated in media both with and without added IL-1.

Intracellular levels of CRP were found to be low in a sample of specimens from 15 patients ranging from 1.7 to 7.7 mcg/mg wet liver (median 3.57; Q1-Q3, 2.58 - 4.8). Complete and instantaneous release of this amount of CRP from a normal liver of 1500 g into a circulating plasma volume of 5 l would result in a concentration of approximately 0.1 mg/l which is well below the concentrations seen with even mild acute phase stimulation, consistent with the known evidence that the rise in concentration of acute phase plasma proteins is due to synthesis of new protein and not to release of intracellular stores.

The levels of CRP in the medium at the end of the incubation period from the 'unstimulated' liver slices (ie those incubated in medium without the addition of exogenous IL-1) was, however, greater than would have been expected if no new synthesis of CRP had taken place. Therefore these apparently unstimulated pieces of liver
had also begun to synthesise CRP. Three sources for inducers are thought possible. Firstly that, before being removed, the liver tissue had been exposed to IL-1 or other cytokines which had been induced by the trauma of laparotomy. Secondly, that cells damaged by the trauma of harvesting the biopsy or by the ischaemia during incubation had activated Kupffer cells within the liver slice to produced new cytokines. Thirdly that the medium bathing the liver slices contained some substance which was activating the acute phase reaction either by a direct action on hepatocytes or indirectly via Kupffer cells.

It is not possible to differentiate in this model which of these is the greatest influence. The likelihood of contaminants in the medium is low in those incubations without FCS where an entirely artificial defined chemical medium was used and where bacterial growth was reduced to undetectable levels by the addition of antibiotics. I conclude that one of the first two mechanisms is therefore more probable.

The term "CRP production" is used to here to indicate the amount of CRP in the medium at the end of the incubation period, and does not allow for newly synthesised CRP not released from hepatocytes or the small amount of pre-existing stored CRP which was released into the medium. However after 24 hours incubation the intracellular CRP concentration was similar to the levels measured in the fresh liver samples, so that the amount of CRP in the medium equates well with the amount of newly
synthesised CRP. This is illustrated for two of the experiments in the figures 19 and 20 on pages 127 and 128.

The sensitivity of the ELISA used to measure CRP was 0.1 mcg/l. The actual concentration of CRP in the media varied between 0.23 and >1000 mcg/l. Values close to the lower limit of the assay were fairly common in phase 1, thus justifying initial fears and the decision to continue incubation for 24 hours. However in phase 2 CRP concentrations in the medium were much greater and a shorter incubation time of say 12 hours which would have reduced the deterioration of the liver slices, would still have produced easily measurable quantities of CRP.

The influence of adding interleukin-1 to the medium

Despite the production of CRP in the 'unstimulated' samples a clear effect of the additional stimulation of exogenous IL-1 was seen both in phase 1 and phase 2. CRP production (that is CRP released into the medium) rose from a median of 7.48 to 11.85 mcg/mg liver in phase 1, and from 93.3 to 137 mcg/mg liver in phase 2, and both of these differences were highly statistically significant (figures 13 and 14, pages 117 and 118).

That the concentration of 10 U/ml IL-1 added was sufficient to produce maximal CRP production is supported by the dose response curves obtained from liver samples in 14 patients. These showed no extra production of CRP when 20 U/ml IL-1 was added to the medium when compared to 10 U/ml. Indeed 5 U/ml induced the synthesis of nearly as much CRP as 10 U/ml (median 161 mcg/mg liver v 192 mcg/mg)
and the difference was not statistically significant. This finding is supported by the work of Le and Mortensen using mouse hepatocyte cultures who noted that the addition of excess concentrations of IL-1 reduced the synthesis of acute phase proteins [Le and Mortensen, 1986].

The effect of increasing the oxygenation of the sample

The effect of changing the concentration of oxygen in which the slices were incubated was quite marked, raising median CRP production some 13 fold from 10.8 to 137 mcg/mg liver (figure 16, page 122). In retrospect this ought to have been carried out from the beginning.

The concentration of oxygen selected for the prolonged preservation of tissue varies with differing tissues. True organ culture, ie. the maintenance of stable functioning solid blocks of tissue, of mammalian liver has not yet been achieved though maintenance of histologically normal liver for up to 6 days has been reported [Campbell and Hales, 1971]. The optimal oxygen concentration appears to be between 60% [Laufs and Walker, 1970] and 95% [Campbell and Hales, 1971]. Concentrations as high as 3 atmospheres have been used but these proved toxic [ibid].

The effect of foetal calf serum (FCS) in the medium

Figure 22, page 131, presents some data which is difficult to interpret. It appears to show that in the absence of FCS in the incubating medium, IL-1 has no stimulating effect on CRP production. FCS in itself did
not affect the level of CRP synthesis, but the two
together had a significant effect greater than neither or
either one alone.

These findings appear to suggest that the FCS was
necessary for IL-1 to exert its effect. One explanation
might be that IL-1 did indeed require to be cleaved to a
smaller fragment to become biologically active in this
model, analogous to its action in muscle proteolysis. As
has been stated in the introduction the smaller fragment
of 4.3 kD was dubbed proteolysis inducing factor
[Dinarello et al, 1984]. Whether cleavage is also
necessary for liver stimulation and whether FCS contains
the necessary proteolytic enzymes to achieve this are
questions that would merit further study. I am not aware
of any evidence in the literature that cleavage is
required for actions other than muscle proteolysis.

Alternatively, FCS may contain a factor or factors
that facilitate protein synthesis and allow IL-1 to act.
The levels of CRP production in the 'unstimulated' liver
slices show that there was in fact considerable
stimulation of the acute phase in these slices too and
this was not enhanced by the presence of FCS. However if
these slices were stimulated by cytokines released from
Kuppfer cells in the specimen, IL-1 might not be the
active cytokine responsible.

Reproducibility

The greatest problem in drawing conclusions from the
results of this arm of the study is the lack of
reproducibility which was observed. Although there was quite good reproducibility between pairs of liver slices processed under identical conditions on the same day from the same patient, there was little concordance between samples from different patients on different days under otherwise apparently identical conditions of incubation. Figure 23 on page 139 shows a correlation between the values for CRP production from 26 paired samples of liver from the same patients. The correlation is close with an Rs value of 0.968. However as can be seen from the graph on page 131 the range of values for CRP production under apparently identical conditions is wide, the largest being approximately 100 times greater than the least. Although differences might have arisen from the ability of the liver of different subjects to produce CRP, these were not reflected in such wide variations in plasma levels seen in the in vivo results. The composition of the medium, the gas phase and other aspects of the incubation were well controlled with little if any variation. The greatest area of variability in the entire process from biopsy to completion was the handling of the liver between its removal from the patient and being placed into the incubator and in particular the slicing process. Differences in traumatic damage by handling and slicing and variations in the thickness of the slices are likely to have influenced the extent and speed of tissue necrosis which occurred during the incubation period which in turn are likely to have been the major determinant of
variability in CRP production. The deficiencies in the use of the McIlwain tissue slicer have been described above. Although when selecting slices for incubation the greatest variability in slice thickness could be avoided by naked eye appearance small differences in slice thickness would make a large difference to the distance that nutrients and oxygen would have to diffuse to reach the innermost cells. Consecutive slices from the same piece of liver tended to be similar in thickness accounting for the consistent results in multiple incubations from the same patient, but variations in the size shape and connective tissue content of different biopsies affected the slice thickness despite the nominally constant increment of advancement of the platter.
4. CONCLUSIONS

In conclusion this study has investigated the relationship between nutritional status and the magnitude of the acute phase plasma protein response by experiments in vivo and in vitro.

The experiments in vivo have demonstrated that patients undergoing surgery for upper gastrointestinal cancer exhibit a typical acute phase reaction but have not been able to demonstrate an influence of the nutritional status of the patients on the magnitude of the acute phase response observed.

In the in vitro experiments a method of incubating human liver slices has been developed and this has been used to demonstrate and measure the synthesis of CRP by human liver tissue. Interleukin-1 has been shown to stimulate the synthesis of CRP by the liver slices.

This arm of the study has also failed to demonstrate a relationship between the nutritional status of the patient from whom the liver sample was taken and the amount of CRP synthesised by the liver sample.

The failure to find a relationship between nutritional status and the acute phase response may have been because malnutrition of the degree seen in these patients is not an influence on the acute phase plasma protein response, the response being preserved because of its central role in repair and recovery after injury, or because the methods employed have not been suitable to demonstrate such an effect. They may have failed to allow
for other influences on the magnitude of the acute phase response or have not had the power to demonstrate it.
5. SUGGESTIONS FOR FURTHER STUDY

I believe that the question of the influence of nutrition on the acute phase reaction has not yet been definitively answered by this study or by previous work. In the in vivo part of this study the number of patients studied is insufficient to be certain that the result is a genuine one. With the slight but definite trends seen by the Milan and Glasgow groups I think it is likely that an effect of nutrition will be demonstrated if a large enough study is undertaken which would overcome the influence of the other variables affecting the magnitude of the acute phase response as discussed earlier. The methodology used in all of these studies including this one is simple and no significant changes require to be made.

The influence of nutritional status on the capacity of liver to synthesise acute phase proteins is still unanswered. I think that the model chosen for this study does offer a suitable means for studying the problem but that more attention has to be paid to achieving greater preservation of the liver tissue and greater reproducibility before a consistent result is likely to emerge. The main areas for improvement are the handling and slicing of the liver prior to incubation and the method of maintaining oxygenation and the supply of nutrients to the liver tissue during the incubation. In addition now that we know that the amount of protein synthesised is sufficiently great that the sensitivity of
the ELISA is not a problem, a shorter period of incubation should be chosen, probably between 12 and 20 hours.

Attempts should be made to obtain a tissue slicer along the lines of that described by Krumdieck, which seems to have significant advantages over the McIlwain machine and with which good results have been described.

Adopting a dynamic organ culture system in which the liver slice and its medium are kept in constant motion would also appear to offer advantages in preservation of the hepatic architecture. Mixing of the medium in this way would maintain the liver in contact with nutrient rich medium and wash away secreted products in a more controlled manner to free diffusion. It also maintains the liver in contact with the medium on both surfaces. In the roller method the liver is placed on the inside of a mesh within a tube and agitated by rollers. Gassing is maintained by way of a gas permeable membrane in the cap of the tube.

These methods could be developed and tested on liver from patients undergoing routine upper abdominal surgery which is freely available, reserving the patients to be studied (the malnourished cancer bearing group) until the method was producing satisfactory results.
APPENDICES
1: THE AMINO ACID SEQUENCE OF C-REACTIVE PROTEIN

PCA-THR-ASP-MET-SER-ARG-LYS-ALA-PHE-VAL-
PHE-PRO-LYS-GLU-SER-ASP-THR-SER-TYR-VAL-
SER-LEU-LYS-ALA-PRO-LEU-THR-LYS-PRO-LEU-
LYS-ALA-PHE-THR-VAL-CYS-LEU-HIS-PHE-TYR-
THR-GLU-LEU-SER-SER-THR-ARG-GLY-TYR-SER-
ILE-PHE-SER-TYR-ALA-THR-LYS-ARG-GLN-ASP-
ASN-GLU-ILE-LEU-PHE-GLU-VAL-PRO-GLU-PHE-
Thr-VAL-ALA-PRO-VAL-HIS-ILE-CYS-THR-SER-
TRP-GLU-SER-ALA-SER-GLY-Ile-VAL-GLU-PHE-
TRP-VAL-ASP-GLY-LYS-PRO-ARG-VAL-ARG-LYS-
SER-LEU-LYS-LYS-GLY-TYR-THR-VAL-GLY-ALA-
GLU-ALA-SER-ILE-ILE-LEU-GLY-GLN-GLU-GLN-
ASP-SER-PHE-GLY-GLY-ASN-PHE-GLU-GLY-SER-
GLN-SER-LEU-VAL-GLY-ASP-ILE-GLY-ASN-VAL-
ASN-MET-TRP-ASP-PHE-VAL-LEU-SER-PRO-ASP-
GLU-ILE-ASN-THR-ILE-TYR-LEU-GLY-GLY-PRO-
PHE-SER-PRO-ASN-VAL-LEU-ASN-TRP-ARG-ALA-
LEU-LYS-TYR-GLU-VAL-GLN-GLY-GLU-VAL-PHE-
THR-LYS-PRO-GLN-LEU-TRP-PRO(COOH)
2: AMINO ACID SEQUENCE OF INTERLEUKIN-1alpha AND beta.

IL-1beta  ALA-PRO-VAL-ARG-SER-LEU-ASN-CYS-THR-LEU-
IL-1alpha ALA-PRO-PHE-SER-PHE-LEU-SER-ASN-VAL-LYS-
ARG-ASP-SER-GLN-GLN-LYS-SER-LEU-VAL-MET-SER-GLY-PRO-TYR-GLU-
TYR-ASN-PHE-MET-ARG-ILE-ILE-LYS-TYR-GLU-PHE-ILE-LEU-ASN-ASP-
LEU-LYS-ALA-LEU-HIS-LEU-GLN-GLN-ASP-MET-GLU-GLN-GLN-VAL-
ALA-LEU-ASN-GLN-SER-ILE-ILE-ARG-ALA-ASN-ASP-GLN-TYR-LEU-THR-
VAL-PHE-SER-MET-SER-PHE-VAL-GLN-GLY-GLU-GLU-SER-ASN-ASP-LYS-
ALA-ALA-ALA-LEU-HIS-ASN-LEU-ASP-GLU-ALA-VAL-LYS-PHE-ASP-MET-
ILE-PRO-VAL-ALA-LEU-GLY-LEU-LYS-GLN-ASN-LEU-THR-LEU-LEU-LEU-
SER-ARG-ILE-SER-LYS-THR-GLN-LEU-TYR-VAL-THR-ALA-GLN-ASP-GLU-
ASP-PHE-asn-lys-Tyr-Pro-Lys-lys-Met-Glu-Lys-Arg-phe-val-
asp-GLN-PRO-VAL-LEU-LEU-lys-Glu-Met-Pro-Glu-Ile-Pro-Lys-
PHE-ASN-lys-Ile-Glu-Ile-Asn-Asn-Lys-Leu-Glu-Phe-Glu-Ser-Ala-
ILE-THR-GLY-SER-GLU-THR-ASN-LEU-LEU-PHE-PHE-TRP-GLU-THR-HIS-
GLN-PHE-PRO-ASN-TRP-TYR-ILE-SER-THR-SER-GLN-ALA-GLU-ASN-MET-
GLY-THR-LYS-ASN-TYR-PHE-THR-SER-VAL-ALA-HIS-PRO-ASN-LEU-PHE-
PRO-VAL-PHE-LEU-GLY-GLY-THR-LYS-GLY-GLY-GLN-ASP-ILE-THR-ASP-
ILE-ALA-THR-LYS-GLN-ASP-THR-TRP-VAL-CYS-LEU-ALA-GLY-GLY-PRO-
PHE-THR-MET-GLN-PHE-VAL-SER-SER
PRO-SER-Ile-THR-asp-phe-GLN-Ile-LEU-GLu-ASN-GLN-ALA
### 3: COMPOSITION OF DULBECCO'S MODIFIED EAGLE MEDIUM

<table>
<thead>
<tr>
<th>Inorganic salts</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>200</td>
</tr>
<tr>
<td>Fe(NO₃)₃·9H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl</td>
<td>400</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>200</td>
</tr>
<tr>
<td>NaCl</td>
<td>6400</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>3700</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>125</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acids</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine·HCl</td>
<td>84</td>
</tr>
<tr>
<td>L-cystine</td>
<td>48</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>580</td>
</tr>
<tr>
<td>Glycine</td>
<td>30</td>
</tr>
<tr>
<td>L-histidine·HCl·H₂O</td>
<td>42</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>105</td>
</tr>
<tr>
<td>L-leucine</td>
<td>105</td>
</tr>
<tr>
<td>L-lysine·HCl</td>
<td>146</td>
</tr>
<tr>
<td>L-methionine</td>
<td>30</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>66</td>
</tr>
<tr>
<td>L-serine</td>
<td>42</td>
</tr>
<tr>
<td>L-threonine</td>
<td>95</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>16</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>72</td>
</tr>
<tr>
<td>L-valine</td>
<td>94</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td>mg/l</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
</tr>
<tr>
<td>D-Ca pantothenate</td>
<td>4</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>4</td>
</tr>
<tr>
<td>Folic acid</td>
<td>4</td>
</tr>
<tr>
<td>i-inositol</td>
<td>7.2</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>4</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>4</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.4</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Other components</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4500</td>
</tr>
<tr>
<td>Phenol red</td>
<td>15</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110</td>
</tr>
</tbody>
</table>
4: THE GLYCOLYTIC PATHWAY AND THE PLACE OF THE THREE MEASURED ENZYMES.

hexokinase →

↓

glucose 6-phosphate

↓

fructose 6-phosphate

phosphofructokinase →

↓

fructose 1,6-diphosphate

phosphate 1,6-diphosphatase →

↓

glyceraldehyde 3-phosphate + dihydroxyacetone phosphate

↓

1,3-diphosphoglycerate

↓

3-phosphoglycerate

↓

2-phosphoglycerate

↓

phosphoenolpyruvate

↓

pyruvate

↓

lactate
Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it.

Dr Johnson

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Plate 1. Photomicrograph of liver from patient no 17 stained with PAS. Appearances of fresh liver prior to incubation. For description see page 136.

Plate 2. Photomicrograph of liver from patient no 17 stained with PAS. Appearances after incubation for two hours. For description see page 136.
Plate 3. Photomicrograph of liver from patient no 17 stained with H & E. Appearances after incubation for four hours. For description see page 136.

Plate 4. Photomicrograph of liver from patient no 17 stained with H & E. Appearances after incubation for six hours. For description see page 137.
Plate 5. Photomicrograph of liver from patient no 17 stained with H & E. Appearances after incubation for 24 hours. For description see page 137.

Plate 6. Photomicrograph of liver from patient no 28 stained with H & E. Appearances before incubation. For description see page 137.
Plate 7. Photomicrograph of liver from patient no 28 stained with H & E. Appearances of fresh liver prior to incubation. For description see page 137.

Plate 8. Photomicrograph of liver from patient no 28 stained with H & E. Appearances after incubation for 18 hours. For description see page 138.
Plate 9. Photomicrograph of liver from patient no 28 stained with H & E. Appearances of edge of specimen after incubation for 24 hours. For description see page 134.

Plate 10. Photomicrograph of liver from patient no 28 stained with H & E. Appearances of periportal area after incubation for 24 hours. For description see page 134.