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A STUDY OF CERTAIN KETO-ACIDS IN METABOLIC DISORDERS, WITH PARTICULAR REFERENCE TO HEPATOCELLULAR DISEASE AND DIABETES MELLITUS.

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Part 1 Introduction.

PART I.

INTRODUCTION.

1. Definition.

The keto acids are those organic acids which contain a carbonyl group in addition to that which is a component part of the carboxyl group, i.e. R.CO.COOH.

2. Biological Importance.

The role of keto-acids as intermediates in the metabolism of carbohydrates, fats and proteins has ensured the interest of biochemists for many years, but the understanding of their importance in the chemistry of pathological processes is only now becoming possible, after their physiological importance has become more fully apparent; much still remains to be done.

During the third decade of the 20th century, intensive work on the aerobic catabolism of carbohydrates was undertaken by many workers, particularly Szent-Györgyi (1935,1936); Stare and Baumann (1936); Martius (1937) and Martius and Knoop (1937); their observations on the catalytic effect of several organic acids on oxygen uptake by minced pigeon muscle were combined with his own results by Krebs in 1937, to be amplified into the now well known tricarboxylic acid cycle, a postulated sequence of events occurring during the aerobic catabolism of carbohydrate (Krebs and Johnson, 1937). Subsequent work by Krebs and by other workers has served to confirm the essentially correct nature of the original concept, although important details were added in later years,

e.g. the importance of co-enzyme A in the metabolic cycle (See Lipmann, 1954).

Further, it is now apparent that the tricarboxylic acid cycle provides the central key to the understanding of the intermediate metabolism of many compounds including the three major groups or "proximate constituents" of the body, i.e. carbohydrates, fats and proteins.

Figure 1 presents a scheme of the main inter-relationships of the compounds of the tricarboxylic acid cycle and between the cycle and other major metabolic pathways; the diagram incorporates extensions made since Krebs' original publication in 1937.

Those compounds of immediate interest in this thesis are marked by an asterisk.

It will be seen that pyruvate, α-ketoglutarate (now sometimes called α-oxoglutarate), oxaloacetate and oxalosuccinate are directly implicated in the cycle; the biological importance of these keto-(oxo-) acids has been reviewed by Krebs (1943) and by Ochoa (1951).

Since the citric acid cycle, is, in most cells, the main source of the adenosine triphosphate (ATP) necessary for the synthesis of essential cellular components including proteins, polysaccharides and other complex molecules, it is not surprising that the enzymes of the cycle have been found in most mammalian tissues which have been studied. It is not proposed to discuss in detail the energy changes accompanying the steps of the tricarboxylic acid cycle, and the reader is referred to the review of F.E. Hunter (1951).

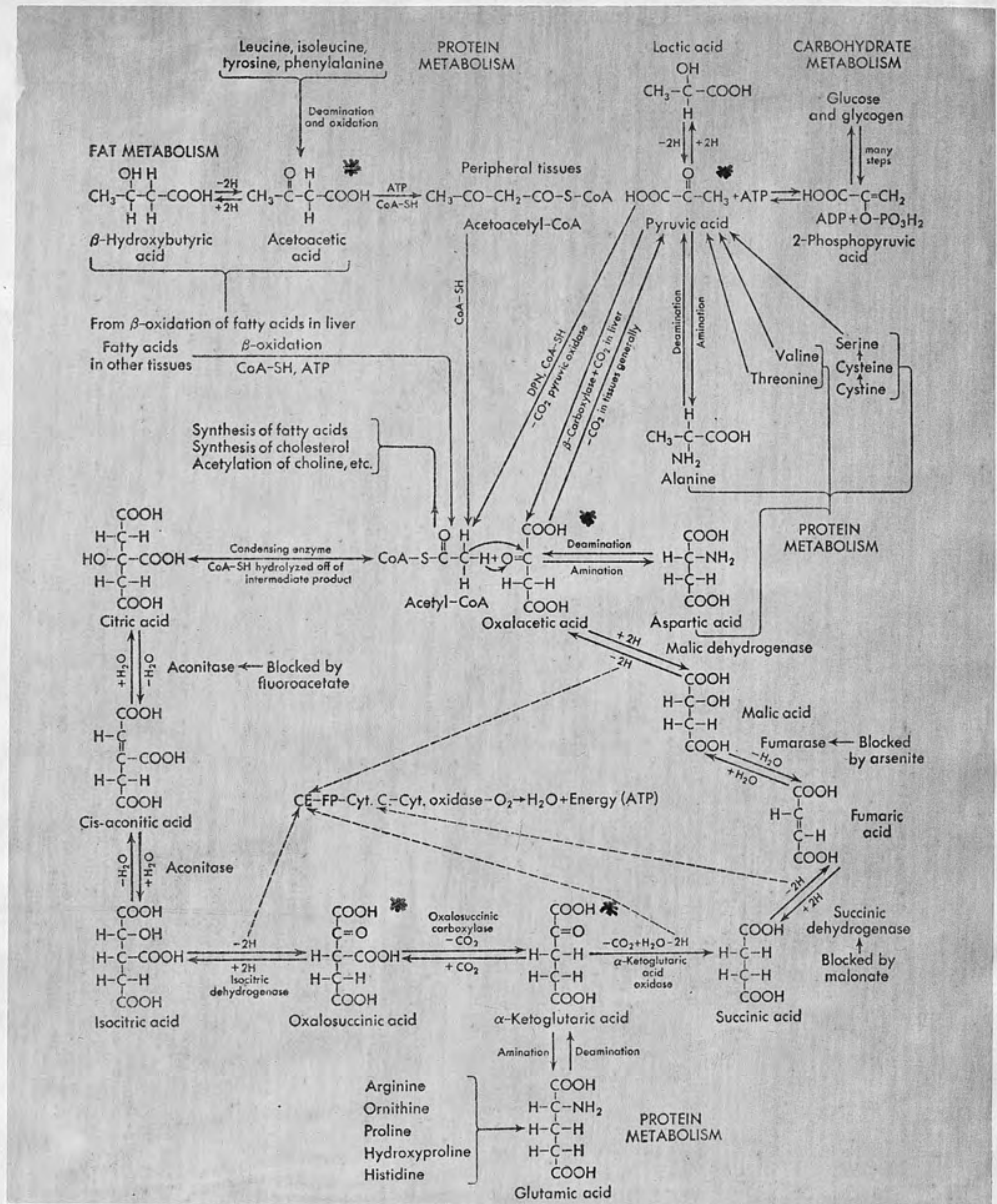


Fig. 1. Schematic integration of carbohydrate, fat and protein metabolism through the tricarboxylic acid cycle (After West and Todd, 1955).

2.1. Carbohydrate Metabolism.

Carbohydrate does not directly enter the citric acid cycle, but is first broken down by glycolysis to form pyruvic acid, which then undergoes oxidative decarboxylation to form acetyl co-enzyme A and enters the citric acid cycle by coupling with oxaloacetate to form citrate (Fig. 2).

Although the glycolytic pathway produces some energy in the form of ATP, it is less efficient than the citric acid cycle. Nevertheless, some mammalian tissues appear to acquire most of their energy from an active glycolytic pathway, rather than from the citric acid cycle, which remains relatively inactive, e.g. retina, embryonic tissue, bone marrow and brain (Warburg, 1930; Quastel, 1939).

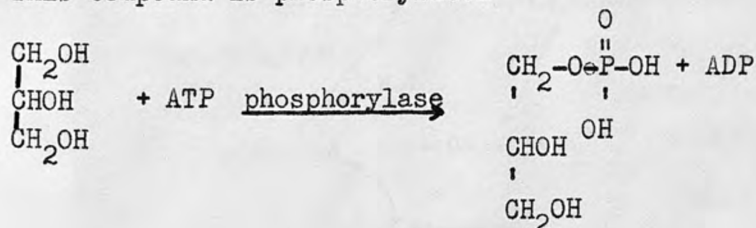
Evidence that the citric acid cycle is concerned in carbohydrate metabolism in invertebrates and plants has also been obtained (Rees, 1953; Vickery and Abrahams, 1949).

2.2. The Catabolism of Fats.

The preliminary steps in the catabolism of triglycerides involves hydrolysis to form glycerol and fatty acids which are then metabolised:

a) Glycerol.

This compound is phosphorylated



α-glyceryl phosphate

and the product is reduced to dihydroxyacetone phosphate, at which stage it enters the glycolytic pathway.

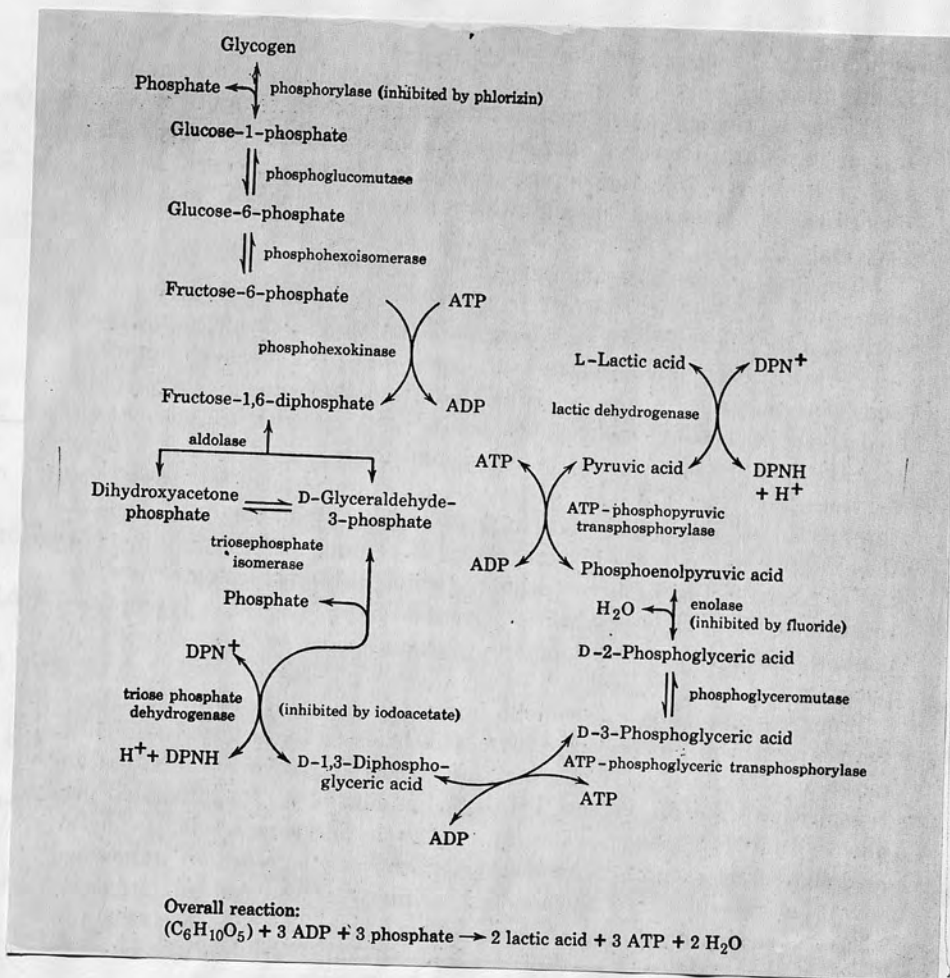


Fig. 2. The glycolytic pathway (Fruton & Simmonds, 1958).

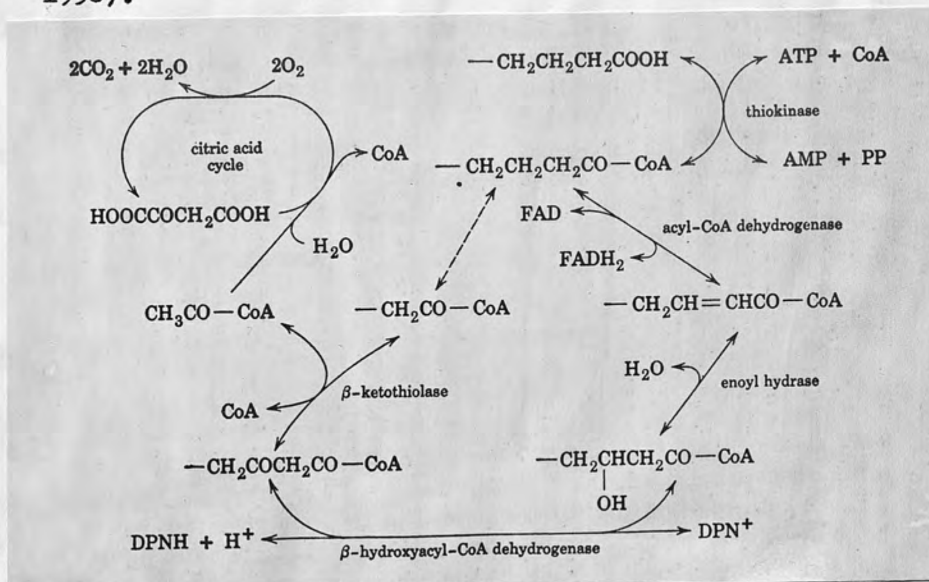
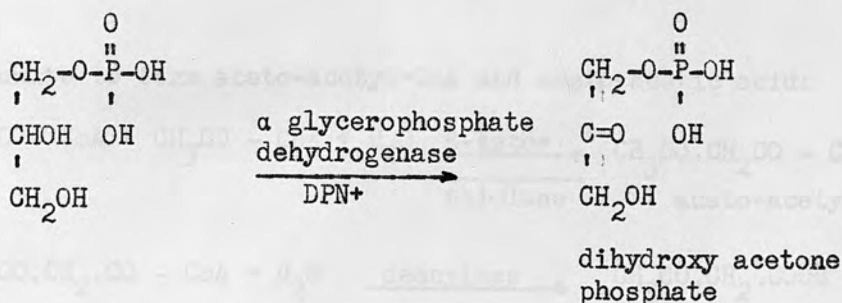


Fig. 3. β-Oxidation of fatty acids (Fruton and Simmonds, 1958)



b) Fatty acids.

It is now generally accepted that the naturally occurring fatty acids are degraded by undergoing β-oxidation. Both saturated and unsaturated fatty acids undergo activation, oxidation, hydration, re-oxidation and splitting serially (see Fig. 3).

The consequence of the cleavage described above is the formation of acetyl-co-enzyme A, which enters the tricarboxylic acid cycle.

In the case of fatty acids containing even numbers of carbon atoms, the molecule is broken down entirely to the two carbon fragments of acetyl-coenzyme A.

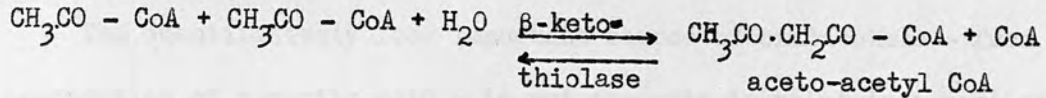
"Odd numbered" fatty acids undergo a similar process, but propionyl - CoA, a residual three carbon fragment, is left. It is, however, easily converted to propionic acid which readily undergoes carboxylation to form succinic acid, the latter entering the tricarboxylic acid cycle directly. (Huennekens, etal, 1951; 1953).

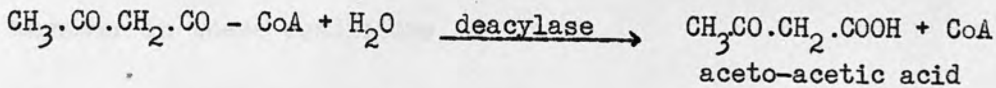
The metabolic changes described above usually occur in the liver, and under normal circumstances, the "2 carbon fragments" are removed via the citric acid cycle as rapidly as they are formed.

c) Fat metabolism in unphysiological conditions.

When the removal of acetyl-CoA is inhibited, two of its molecules

may unite to form aceto-acetyl-CoA and aceto-acetic acid:


$$CH_3CO - CoA + CH_3CO - CoA + H_2O \underset{\text{thiolase}}{\overset{\beta\text{-keto-}}{\rightleftharpoons}} CH_3CO.CH_2CO - CoA + CoA$$
aceto-acetyl CoA


$$CH_3.CO.CH_2.CO - CoA + H_2O \xrightarrow{\text{deacylase}} CH_3CO.CH_2.COOH + CoA$$
aceto-acetic acid

Liver does not possess an aceto-acetate activating system and aceto-acetic acid, with two of its metabolic products leak into the blood:

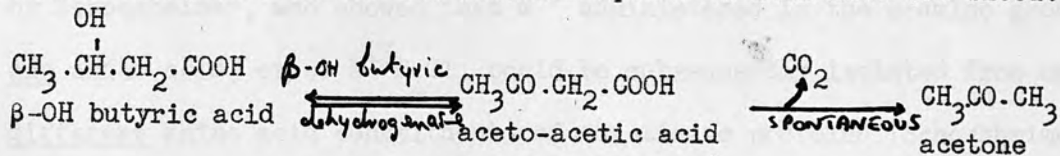

$$\underset{\beta\text{-OH butyric acid}}{CH_3.CH(OH).CH_2.COOH} \underset{\text{dehydrogenase}}{\overset{\beta\text{-OH butyric}}{\rightleftharpoons}} \underset{\text{aceto-acetic acid}}{CH_3CO.CH_2.COOH} \xrightarrow[\text{SPONTANEOUS}]{CO_2} \underset{\text{acetone}}{CH_3CO.CH_3}$$

Fig. 4. The interrelationship of "ketone bodies".

There are four conditions in which ketonaemia may occur:

1) Poisoning with malonate causes a block of the citric acid cycle, acetyl-CoA accumulates, and the formation of "ketone bodies" increases (Geyer and Cunningham, 1950).

2. Excess NH_3 added to rat liver homogenates results in reductive amination of α-ketoglutarate to form glutamate; the interruption of the citric acid cycle causes a rise in "ketone body" formation due to accumulation of acetyl-CoA (Recknagel and Potter, 1951).

3 and 4. Starvation and diabetic crisis cause ketonaemia, and in both appears to be due to a deficiency of oxalacetate, which is normally provided by the carboxylation of pyruvate. In starvation, lack of carbohydrate causes a reduction in available pyruvate, whilst impaired glycolysis in diabetes has the same effect. Thus acetyl-CoA has an inadequate supply of oxalacetate to condense with at its site of entry

into the tricarboxylic acid cycle, and ketonaemia results.

The quantitatively less important source of oxalacetate - from the deamination of aspartic acid - is not adequate to maintain normal catabolic turnover in these conditions.

2.3 The catabolism of Amino Acids.

The lability of the amino group of the α-amino acids was demonstrated by Schoenheimer, who showed that N^{15} administered in the α-amino group of one amino acid, or as $N^{15}H_4Cl$, could be subsequently isolated from many different amino acid constituents of the tissue proteins (Schoenheimer et al, 1939).

Further, it was shown that certain indispensable amino acids (e.g. tryptophan and histidine) could be replaced in the diet of a growing rat by the corresponding α-keto acids. This type of work, and the number of "substitutes" for indispensable dietary amino acids is comprehensively reviewed by Jackson and Chandler (1939).

a) Oxidative Deamination. The amino acids may lose their $\alpha\text{-}NH_2$ group by oxidation:

$$H_2N.\overset{R}{\overset{|}{C}H}.COOH + \tfrac{1}{2}O_2 \longrightarrow O = \overset{R}{\overset{|}{C}}.COOH + NH_3$$

and the presence of both a D- and an L- amino acid oxidase in rat kidney was demonstrated by Krebs (1935). He found that the L-amino acid oxidase activity was associated with insoluble tissue particles, from which it could not be separated. The D-amino acid oxidase, on the other hand, was soluble.

Blanchard et al (1944, 1945), however, isolated soluble enzyme

TABLE 1.

Mammalian Enzymes Catalysing Oxidative Deamination of Amino Acids

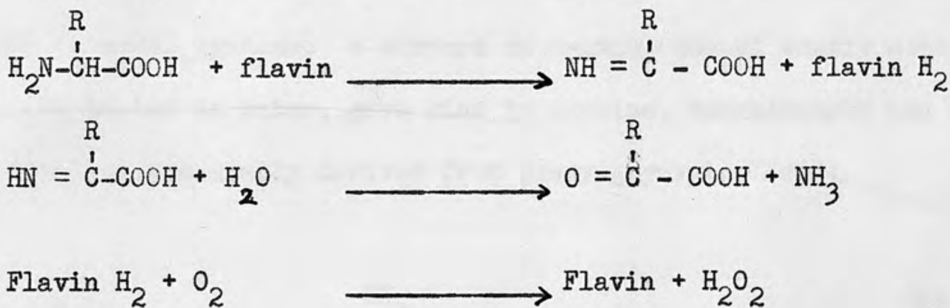
Types of enzyme	Prosthetic group	Sources	Substrate(s)	Product(s)	Author
1. D-amino acid oxidase	FAD	Liver, kidney	All D-amino acids except glycine & glutamic acid	Corresponding keto-acids	Warburg and Christian, 1938
2. L-amino acid oxidase	FMN	Liver, kidney	All L-monoamino, monocarboxylic acids except glycine and those containing a hydroxyl group. No effect on dicarboxylic or diamino amino acids	Corresponding keto-acids	Blanchard et al, 1944, 1945.
3. Glycine oxidase	FAD	Liver, kidney	Glycine	Glyoxylic acid	Ratner, 1944. et al
4. Glutamic dehydrogenase	None. (coenzyme DPN or TPN)	Liver, kidney	Glutamic acid	α-ketoglutaric acid	Olsen and Anfinsen, 1952, 1953.

preparation from rat liver and kidney, which specifically oxidised L-amino acids.

It is an interesting paradox that although D-amino acid oxidase is widely distributed both in mammals and micro-organisms, and has a turnover number of 2,000 as opposed to a turnover number of 6 for the mammalian L-amino acid oxidase, no apparent physiological rôle for the former enzyme has yet been found.

It is suggested by West and Todd (1955b) that the D-enzyme may serve to destroy D-amino acids produced in some metabolic process, but this must remain conjectural, since no D-enantiomorph has been isolated from mammalian sources.

The flavoprotein enzymes may use molecular oxygen as the ultimate electron acceptor (see Fruton and Simmonds, 1958).

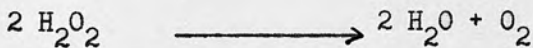


$$H_2N\text{-}\overset{R}{\overset{|}{C}}H\text{-}COOH + \text{flavin} \longrightarrow NH = \overset{R}{\overset{|}{C}} - COOH + \text{flavin } H_2$$

$$HN = \overset{R}{\overset{|}{C}}\text{-}COOH + H_2O \longrightarrow O = \overset{R}{\overset{|}{C}} - COOH + NH_3$$

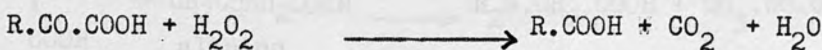
$$\text{Flavin } H_2 + O_2 \longrightarrow \text{Flavin} + H_2O_2$$

In vivo, it is probable that the widely distributed enzyme catalase, immediately catalyses the decomposition of the hydrogen peroxide



$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

In vitro, however, the keto-acids produced in the above reaction is immediately oxidised to the next lower fatty acid, unless some substance is added which is preferentially oxidised by the hydrogen peroxide (e.g. ethanol).



$$R.CO.COOH + H_2O_2 \longrightarrow R.COOH + CO_2 + H_2O$$

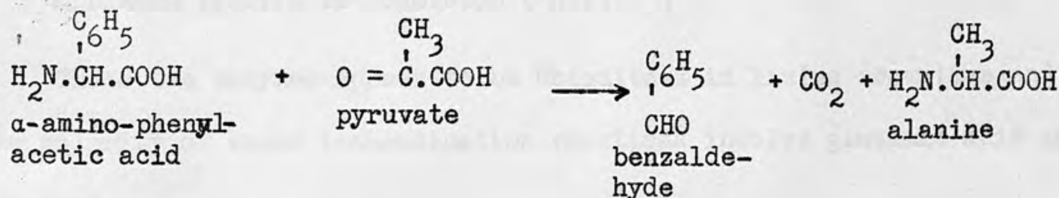
This observation is of considerable practical importance, for the oxidases have been used to isolate one enantiomorph from a mixture of both, by destroying the unwanted amino acid.

Meister (1951, 1952) has used an L-amino acid oxidase obtained from snake venom, to prepare several α-keto-acids from the corresponding amino acids.

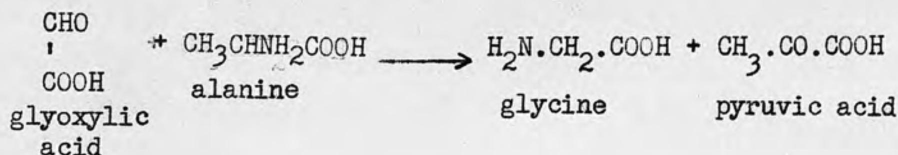
The snake venom enzyme was used because its turnover number is 3,100 as apposed to 6 for the enzyme from mammalian sources.

b) Transamination. Although the amino acids may lose their α-NH_2 group by enzymic oxidation, it now seems likely that deamination occurs *in vivo* predominantly as the result of the action of transaminases which are widely distributed.

Transamination was first noticed incidentally by Herbst and Engel (1934) in model systems: a mixture of α-amino-phenyl acetic acid and pyruvate heated in water, gave rise to alanine, benzaldehyde and CO_2, the last two presumably derived from phenylglyoxylic acid:



More recently non-enzymatic transamination between glyoxylic acid and alanine, aspartate or glutamic acid at pH 7.4 and 25-30°C has been observed, with the production of glycine and the corresponding keto-acids (Nakada and Weinhouse, 1953).



Giri (1954) and Giri and Kalyankar (1953) noted transamination phenomena when they studied the chromatographic separation of the free keto-acids in tissue extracts. It appeared that the chromatographic paper itself catalysed transfer of α-amino groups from the amino acids present to pyruvate or α-ketoglutarate, when heated to 80°C, thus causing losses of these keto-acids.

The first evidence for enzymic transamination was presented by Braunstein and Kritzmann(1937) who showed that minced pigeon breast muscle catalysed the transfer of the α-NH_2 group of any amino acid except glycine to α-ketoglutarate or oxalacetate, producing L-glutamic or L-aspartic acid respectively.

Cohen later (1939,1940) showed the existence of only two transaminase systems in pig heart muscle:

1. L-glutamic acid + oxalacetic acid = α-ketoglutaric acid + L-aspartic acid.

   GLUTAMIC OXALO-ACETATE TRANSAMINASE ("G.O.T.")

2. L-glutamic acid + pyruvic acid = α-ketoglutaric acid + L-alanine

   GLUTAMIC PYRUVIC TRANSAMINASE ("G.P.T.")

These two enzymes appear to be ubiquitous in living organisms and the majority of known transamination reactions involve glutamic acid as a reactant.

Nevertheless, it is now known that G.O.T. and G.P.T. are only two of a wide variety of transamination reactions in biological systems.

Table 2. General Types of Transamination Reactions.

Type	General Reaction	Tissue of origin
1	L-α amino acid + α-ketoglutarate ⇌ α-ketoacid + L-glutamic acid	Generalised in tissues
2	γ-amino-butyric acid + α-ketoglutarate ⇌ succinic semi-aldehyde + L-glutamic acid	Brain
3	a) L-glutamine + α-keto acid ⇌ α-ketoglutaramic acid + L-α amino acid	Liver
	b) L-aspargine + α-keto acid ⇌ α-ketosuccinic acid + L-α amino acid	Liver
4	L-α amino monocarboxylic $acid_1$ + α-keto monocarboxylic acid 2 ⇅ α-keto-monocarboxylic $acid_1$ + L-α amino monocarboxylic $acid_2$	Liver

These transaminase systems have been widely studied by Meister and reviewed by him (Meister, 1955).

The two type 1 enzymes, G.O.T. and G.P.T., are very widely distributed in the human body, and damage to some organs, particularly heart and liver, may cause an elevation of their activity in serum. The value of estimating the serum G.O.T. and G.P.T. in the study of disease is well reviewed by Wróblewski (1958).

Transaminases require pyridoxal phosphate as co-enzyme. (Fig. 5).

It is postulated that the amino group donated by $HOOC.\overset{R1}{\overset{|}{C}}H.NH_2$ is transferred to the acceptor keto acid $HOOC.\overset{R2}{\overset{|}{C}}.= O$ viâ pyridoxamine phosphate and by a reversal of the reactions shown in Fig. 5. (Peterson and Sober, 1954; Meister, 1954, 1954; Olivard and Snell, 1955).

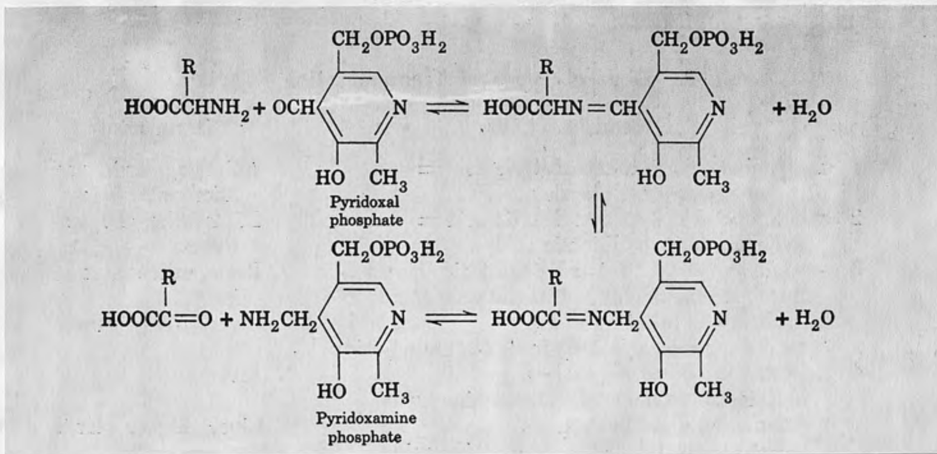
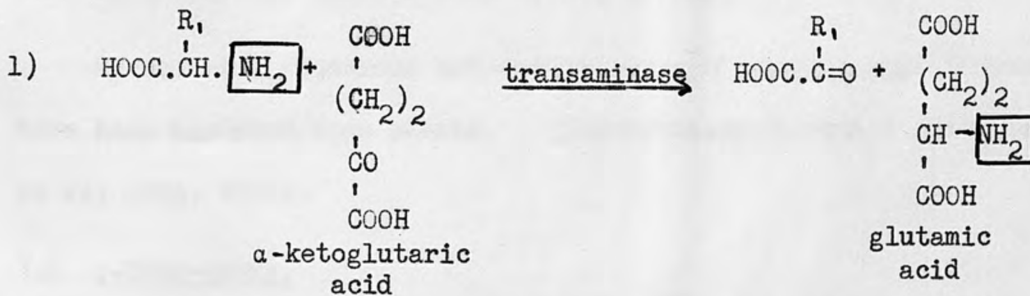
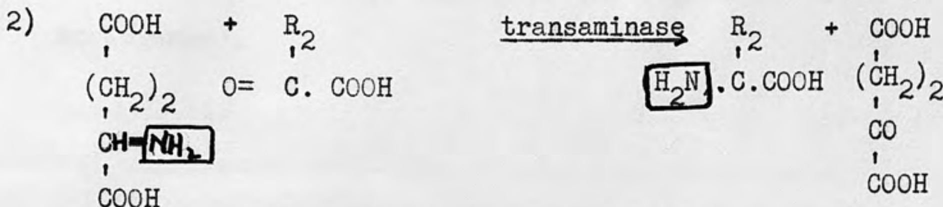
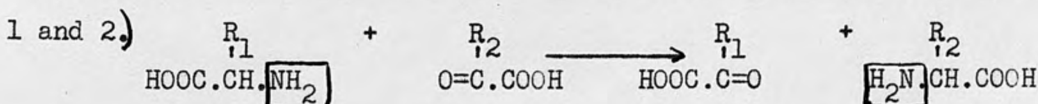


Fig. 5. Role of pyridoxine in transaminase systems (After Fruton and Simmonds, 1958).

In vitro studies by Snell (1945) suggest that the reactions of figure 5 are freely reversible , so that it is easy to see how the α amino group from one amino acid may be utilised to synthesise another amino acid, if the keto-acid carbon skeleton of the latter is available.







The particular importance of glutamic and aspartic acids in nitrogen transfer is in agreement with the observations that they take up N^{15} administered to rats as labelled amino acids more rapidly than any of the other tissue amino acids (Schoenheimer et al, 1939).

Apart from the preliminary deamination which is common to all α-amino acids, each one has its own particular catabolic pathway, some of which are complex.

However, degradation eventually gives rise to a compound related to the substrates of the citric acid cycle, and their relationship to this cycle are summarised in Figure 1.

3. Keto-acids isolated from mammalian sources.

Althouth it is evident from the above discussion that many keto-acids of great physiological significance exist, relatively few have been isolated from mammalian blood and urine.

By contrast, numerous keto-acids, many of obscure significance, have been isolated from plants. (Towers&Steward,1954); Virtanen et al, 1954, 1955).

3.1 α-Keto-acids.

These compounds are presented below in tabular arrangement.

Table 3. α-keto-acids isolated from mammalian sources.

Acid	Abbreviation	Formula	Source
Pyruvic	P	$CH_3CO.COOH$	Blood, urine, C.S.F.
α-keto-glutaric	KG	$HOOC.CO.(CH_2)_2COOH$	Blood, urine, C.S.F.
α-keto-isovaleric	KIV	$(CH_3)_2CH.CO.COOH$	Blood, Urine, only in "Maple Syrup" Disease
α-keto-isocaproic	KIC	$(CH_3)_2CH.CH_2CO.COOH$	as KIV
α-keto-β-methyl-isovaleric	KMV	$CH_3CH_2CH(CH_3)CO.COOH$	as KIV
Phenyl-pyruvic	PP	$C_6H_5CH_2CO.COOH$	Blood and urine, ONLY in phenylketonuria.

3.2 β-keto acids.

Table 4. β-keto acids of physiological importance.

Acid	Abbreviation	Formula
Oxalo-acetic	OA	$HOOC.CH_2.CO.COOH$
Oxalosuccinic	OS	$HOOC.CH_2.CH(COOH)CO.COOH$
Aceto-acetic	Ac.Ac.	$CH_3CO.CH_2COOH$

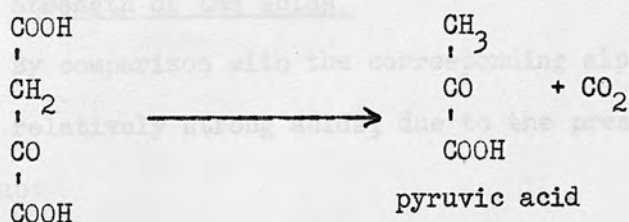
OA and OS have not been isolated from mammalian blood, although Frohman, Orten and Smith (1951) claimed to have isolated OA from rat blood (<0.1 mg/100 ml.). Their experimental method has been severely criticised by several workers (see Neish, 1957) and no one appears to have substantiated their claim.

The elusive nature of OA and OS is probably explained by the observations of Krebs (1942) and later by Ochoa (1948).

Krebs found that the compounds underwent spontaneous decarboxylation in pure aqueous solution, and that decomposition was accelerated by trace amounts of some amines (e.g. co-carboxylase) and of the polyvalent cations Co^{++}, Zn^{++}, Cu^{++}, Fe^{++} and Fe^{+++}, although Ca^{++}, Ba^{++}, Mg^{++} and Mn^{++} were relatively ineffective.

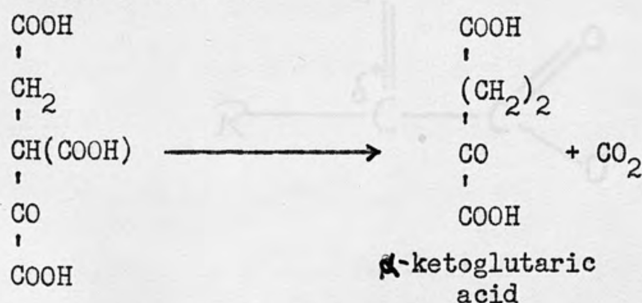
Ochoa made quantitative estimates of the rate of decomposition of OA and OS.

1. <u>Oxalacetate.</u>



Half life: 216 min. (pH 5.0, 25°C).

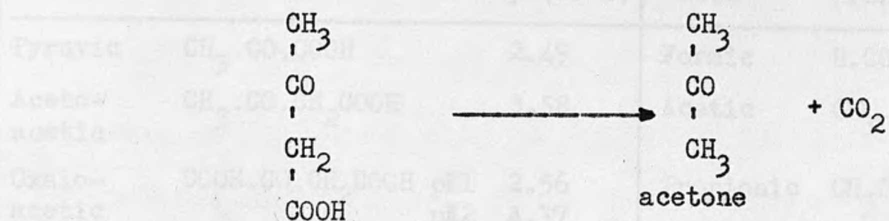
2. <u>Oxalosuccinate.</u>



Half life: 25 min. (pH 5.1, 25°C).

Ostern (1933) and Bessmann and Layne (1950) found that amino acids also catalysed the decomposition of OA and OS.

Aceto-acetic acid is less unstable than OA and OS, but it will nevertheless decompose slowly in aqueous solution.

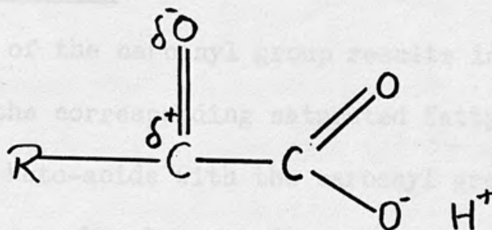


4. Properties of the keto-acids.

The presence of the carbonyl group endows keto-acids with two important properties.

4.1 Strength of the acids

By comparison with the corresponding alphatic acids, the keto-acids are relatively strong acids, due to the presence of the polar carbonyl group:



(After Fieser and Fieser,1950)

and the published pK values for various acids may be compared:

Table 4. The strength of keto-acids.

Keto-acids			Non substituted acids.		
Acid	Formula	pK(25°C)	Acid	Formula	pK(25°C)
Pyruvic	$CH_3.CO.COOH$	2.49	Formic	$H.COOH$	3.77
Aceto-acetic	$CH_3.CO.CH_2COOH$	3.58	Acetic	$CH_3.COOH$	4.76
Oxalo-acetic	$COOH.CO.CH_2COOH$	pK1 2.56 pK2 4.37	Propionic	CH_3CH_2COOH	4.88
			n-butyric	$CH_3(CH_2)_2COOH$	4.82
α-keto-β-methyl valeric	$CH_3CH_2CH(CH_3)CO.COOH$	2.80			

(Long, King and Sperry, 1961).

It will be noted that the closer the proximity of the carbonyl group to the carboxyl group, the greater is the strength of the acid. The influence of the carbonyl group and of its position on strength is reviewed by Edsall and Wyman (1958).

4.2 Enhanced reactivity.

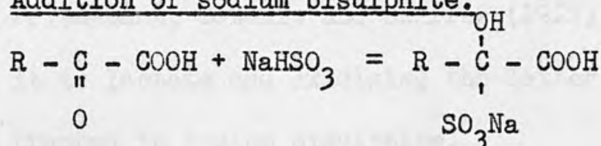
The presence of the carbonyl group results in much greater reactivity than is found in the corresponding saturated fatty acid; indeed as is seen above, those keto-acids with the carbonyl group lying in a β-position with respect to the carboxyl group decompose spontaneously.

In addition to possessing the general features of organic acids, the keto acids react readily with "ketone reagents", and the carbonyl group is readily oxidised or reduced.

Unfortunately these reactions are common to all keto-acids, and though they have been made use of to estimate individual keto-acids, it has, so far, been impossible to separate and identify mixtures of them by purely chemical means.

Many of the methods described below are now obsolete since recent developments, particularly in chromatography, have made it possible to achieve separation and estimation with ease and specificity. However, a few of the older methods have been resuscitated and successfully employed in the solution of specific experimental problems.

4.2.1. Addition of sodium bisulphite.

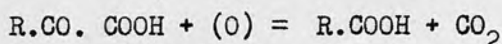


Binding of $NaHSO_3$ with ketones was first introduced by Ripper (1900) to estimate acetaldehyde, but it was subsequently developed for the estimation of any keto-acid (Cook, 1930; Clift and Cook, 1932).

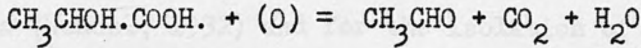
Various authors, however, appear to have found the method unreliable, and it fell into disrepute for the analysis of tissue keto-acids (Long, 1942). Nevertheless, the method has been used successfully to trap and separate α-keto acids from radioactive acetate in metabolic studies using tissue extracts (Kvamme and Hellman, 1954).

4.2.2. Oxidation.

Quantitative conversion to the next lower fatty acid occurs readily and Krebs (1938) successfully exploited this property in tissue studies, measuring the CO_2 evolved manometrically:



Suitable oxidising agents are H_2O_2, $KMnO_4$ (Krebs, 1938; Krebs and Eggleston, 1948; Green, Leloir and Nocito, 1945), or ceric sulphate (Meister, 1952; Fromageot and Desnuele, 1935). Unfortunately, not only does this method estimate <u>all</u> the keto-acids in a tissue extract, but lactate is also oxidised to acetaldehyde, with the production of CO_2:-

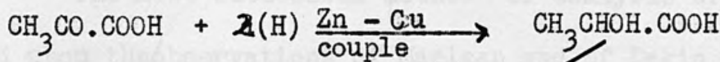


$$CH_3CHOH.COOH. + (O) = CH_3CHO + CO_2 + H_2O$$

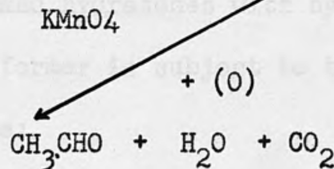
(Gordon and Quastel, 1939).

4.2.3. <u>Reduction.</u>

This also readily occurs, to form the corresponding hydroxy-acid and Friedemann, Cotonio and Shaffer (1927) estimated pyruvate by reducing it to lactate and oxidising the latter to acetaldehyde which was then trapped in sodium bisulphite.



$$CH_3CO.COOH + 2(H) \xrightarrow[\text{couple}]{\text{Zn - Cu}} CH_3CHOH.COOH$$

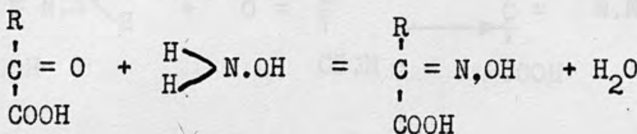


$$CH_3CHO + H_2O + CO_2$$

4.2.4. <u>Condensation Reactions.</u>

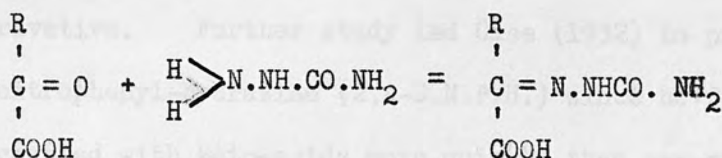
Various condensations, notably with various derivatives of ammonia have been described:

4.2.4.1 <u>with Hydroxylamine.</u>



$$\begin{matrix} R \\ C = O \\ COOH \end{matrix} + \begin{matrix} H \\ H \end{matrix} > N.OH = \begin{matrix} R \\ C = N.OH \\ COOH \end{matrix} + H_2O$$

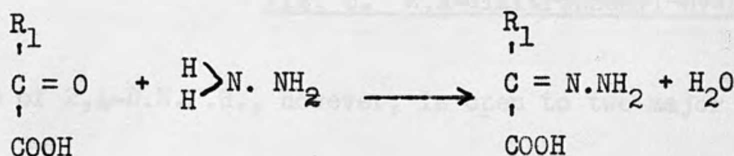
4.2.4.2. With Semicarbazide.

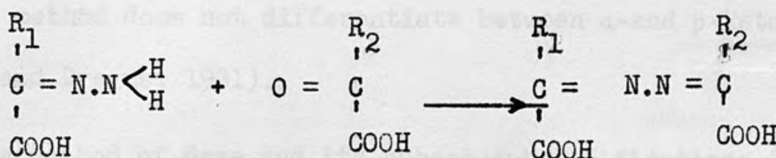


The reaction with semicarbazide has been used to estimate keto-acids (Wendel, 1932) and for the isolation of α-keto-glutarate from kidney homogenates in metabolic studies (Buchanan et al, 1945). The compound has been used as a locating agent when chromatography of free keto-acids has been undertaken, the condensation product being opaque to ultraviolet light (Magasanik and Umbarger, 1950 ; Umbarger and Magasanik, 1952).

4.2.4.3. Hydrazine.

The most successful methods of analysis of the keto-acids is based upon the observations of Maclean and of Dakin and Dudley in 1913 that the keto-acids formed hydrazones with hydrazine and with 4-nitrophenyl-hydrazine. The former is subject to the objection that double condensation occurs:





and for this reason Dakin and Dudley introduced the use of the nitrophenyl derivative. Further study led Case (1932) to propose the use of 2,4 dinitrophenyl-hydrazine (2,4-D.N.P.H.) since he found that this compound coupled with keto-acids more quickly than any other phenylhydrazine derivative.

Case's reagent also had the advantage that the condensation product had an intense yellow colour which became an intense red colour (Max. absorption at approximately 440 mμ) on adding sodium hydroxide. These properties permitted the measurement of very small quantities of keto-acids.

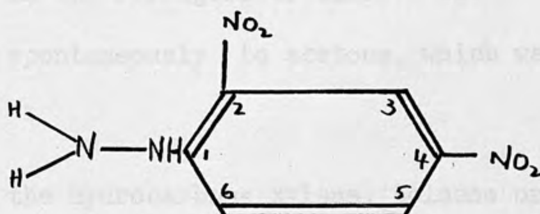


Fig. 6. 2,4-dinitrophenyl-hydrazine.

The use of 2,4-D.N.P.H., however, is open to two major objections:

1) The reagent is non-specific, reacting with any ketone.

2) The method does not differentiate between α-and β-keto-acids (Barrenscheen and Dregus, 1931).

The method of Case and its subsequent modifications (Peters and Thompson, 1934; Lu, 1939; Beuding and Wortis, 1940), attempted to separate the "neutral" and "acidic" hydrazones by partitioning these

compounds between ethyl acetate (containing the non-acidic compounds) and a weak alkali (usually 10% Na_2CO_3 solution) which held the acidic compounds. This method was fairly successful in achieving this aim, but suffered the disadvantage that all keto-acids were quoted as "pyruvate", although, as will be shown below, some of the keto acid present was in the form of α-ketoglutarate. Further, although little the β-keto-acid, aceto-acetic, normally passed into the alkaline layer, sufficient could be extracted into the latter to cause abnormally high levels of "pyruvate" when subjects were ketonaemic.

The first step towards a specific method was made by Friedemann and Haugen in 1943. They found that keeping samples of blood, precipitated with trichloracetic acid, overnight in the refrigerator allowed the aceto-acetic acid to decarboxylate spontaneously to acetone, which was not extracted into the 10% Na_2CO_3.

These authors also noted that the hydrocarbons xylene, toluene or benzene extracted different keto-acid hydrazones to degrees which differed from those of ethyl acetate:

Solvent	Percentage of known K.A. hydrazone content extracted into solvent.	
	Pyruvate	α-Ketoglutarate.
Ethyl acetate	81	79
Toluene	93	29

Thus by using toluene or the other hydrocarbons, specificity was enhanced considerably. Studies of the reaction times between 2,4-D.N.P.H. and pyruvic and α-ketoglutaric acids showed that the former reaction was completed in 5 minutes, the latter requiring 25 minutes.

Based on these observations, two extraction procedures were evolved:

	Procedure A.	Procedure B.
Incubation of reaction mixture at 25°C.	25 min.	5 min
Extraction; volume and solvent	8 ml. ethyl acetate	3 ml. xylene, toluene or benzene
Re-extraction with 10% Na_2CO_3	6 ml.	6 ml.
Alkali added to 5.0 ml. of Na_2CO_3 extract	5.0 ml. x 1.5 N. Na OH	5.0 ml. x 1.5 N.NaOH
Light filter No.	540,420,400	520
Keto acids determined	total	pyruvic acid with 10% of α-ketoglutarate present.

Pure solutions of pyruvic and α-ketoglutaric acids subjected to procedure A were found to possess slightly different light absorption curves, when examined with the Evelyn colorimeter, and the authors exploited these differences to calculate the approximate concentration of each of the keto-acids.

After applying the methods outlined above, Friedemann and Haugen drew the conclusion that some 10-20% of the total keto-acid content of the blood was α-ketoglutarate.

Truly specific methods for the separation of mixtures of ketoacids or their derivations did not become available until the introduction of partition chromatography by Martin (Martin and Synge, 1941; Consden, Gordon and Martin, 1944).

5. Paper Chromatography of Keto-acids.

5.1. The Hydrazones.

Separation of the 2,4-dinitrophenyl-hydrazones of keto-acids by paper chromatography was introduced by Cavallini, Frontali and Toschi (1949a, 1949b) and subsequently modified by various workers (Kulonen et al, 1952; El Hawary and Thompson, 1953; Gey, 1954; McArdle, 1957).

All these methods were essentially similar; reaction mixtures of 2,4-D.N.P.H. and protein free supernatants were extracted after the method of Friedemann and Haugen (1943) using ethyl acetate and 10% Na_2CO_3. The Na_2CO_3 extract was acidified with concentrated HCl and re-extracted into ethyl acetate, which was then evaporated to dryness. The residue was taken up in buffer, pH 7.2, and spotted onto the chromatography paper.

These authors identified α ketoglutarate and pyruvate whilst some thought that substantial quantities of aceto-acetate were present in normal blood (e.g. El Hawary and Thompson, 1953). In fact, this last observation was an artefact and due to the authors being unaware that pyruvate produces two hydrazone spots on chromatography and that one of the pyruvate spots had the same Rf as aceto-acetic hydrazone in some solvent systems (Kulonen, Cárpen and Ruikolainen, 1952; El Hawary and Thompson, 1953, 1954), but not apparently in others (Markees and Gey, 1953; Markees, 1954).

Isomerisation of the keto-acid hydrazones is reviewed below.

Friedemann and Haugen (1943) noted that diethyl ether extracted more of the hydrazones from aqueous solutions than did ethyl acetate,

and Meister (1951) and Metzler and Snell (1952b) pointed out that 10% Na_2CO_2 did not extract all keto-acid hydrazones from ethyl acetate, citing the example of KMV-hydrazone,which is almost entirely retained in the Na_2CO_3 solution.

In 1954, Cavallini and Frontali suggested that ether be used as the extracting solvent, and that the dry residue obtained by evaporating this solution be partitioned between chloroform and I.O.N. NH_4OH solution.

The adoption of this method was quickly followed by the identification of α-keto-isocaproic acid in blood (Biserte, Osteux and Dassonville, 1955), though Turnock (1953) had previously wrongly identified the compound as phenyl-pyruvic acid.

De Schepper, Parmentier and Van der Haeghe (1958) using a similar technique, first identified the three "branch chain" keto-acids (KIV, KIC and KMV) in blood extracts. The hydrazones of these three compounds ran together in a composite spot, and could not be separated by simple modifications of the solvent systems used; the presence of the individual hydrazones within the spot was confirmed by catalytic hydrogenation.

5.2 Identification of the hydrazones of keto acids.

Catalytic hydrogenation of keto-acid hydrazones was first demonstrated by Fisher and Groh (1911), though Towers et al (1954a; 1954b) carried out more detailed studies. The method has been reviewed by Smith (1960) and is generally regarded as a most useful means of identification. Unfortunately there are three defects:

1) A few hydrazones give rise to trace amounts of amino acids other than that corresponding to the keto-acid of origin. Thus Towers et al (1954a) found that reduction of α-ketoglutaric hydrazone gave rise to glutamic acid, and also to minute amounts of γ-amino butyric acid,

thought to be derived from the α-ketoglutarate hydrazone by decarboxylation.

2) Quantitative conversion does not occur. Thus, if a keto-acid produces two spots on chromatography (e.g. P and KIC), only one will reduce to the corresponding amino acid.

3) Reduction of a contaminant commonly present in dinitrophenylhydrazine solutions produce a ninhydrin positive spot. The contaminant is thought to be 1-hydroxy-6-nitro-1,2,3 benzotriazole. (Towers and Mortimer, 1954; Brady and Day, 1923).

5.3. Disadvantages of chromatography of hydrazones.

Although much information about the keto-acids has been obtained by the chromatography of their hydrazones, two major defects are inherent in the method:

1) Formation of Isomers.

Le Page (1950) first noted the formation of two bands from pyruvate hydrazones during column chromatography, and his observations were extended by many other workers:

Authors	Keto-acids producing two spots
Le Page (1950))	Pyruvate
Seligson & Shapiro (1952))	
Markees & Gey (1953))	
Altmann et al (1951))	Phenyl-pyruvate
Kulonen et al (1952))	
Turnock (1953))	
Metzler et al (1954)	Ketoisocaproate; p-hydroxy phenyl-pyruvate

Both Le Page and Metzler and Snell (1952a) reported that fresh ethyl acetate solutions of pyruvic hydrazone gave one spot on chromatography, but if the solution was allowed to stand for some time, two spots were formed.

Cavallini and Frontali (1954) found that synthetic pyruvic hydrazone spotted onto the paper direct and subjected to chromatography produced one spot, but the same keto-acid subjected to their reaction and extraction procedure produced two spots on chromatography.

Metzler and Snell (1952a) and Cavallini and Frontali (1954) reported that the slower of the two spots, when obtained, possessed the same Rf value as the one spot obtained from the chromatography of the fresh synthetic hydrazone.

Markees and Gey (1953) studied the two spots from pyruvate hydrazone in more detail:

Hydrazone	Rf*	max.mμ	M.P.(°C)
"A"	0.36	355	210-211 (sharp)
"B"	0.57	375	Sintered 160,melted 190

* The solvent system used was n-Butanol: 3% NH_4OH = 95:5.

Compound "B" was found to be unstable, forming a mixture of "A" and "B" on standing.

Compound "A" showed the elemental analysis which was anticipated, but,of compound "B", the authors state "Extensive efforts to identify its chemical nature were unsuccessful, and the question of possible isomery, tautomery or transformation into closely associated product must remain open."

Isherwood and Jones (1955), after surveying the physical properties and infra red spectra of the two spots obtained, suggested that isomerisation occurred around the -C= N- bond: (Fig. 7)

McArdle (1957) found that the faster spot was slow to develop the usual pink colour on adding NaOH.

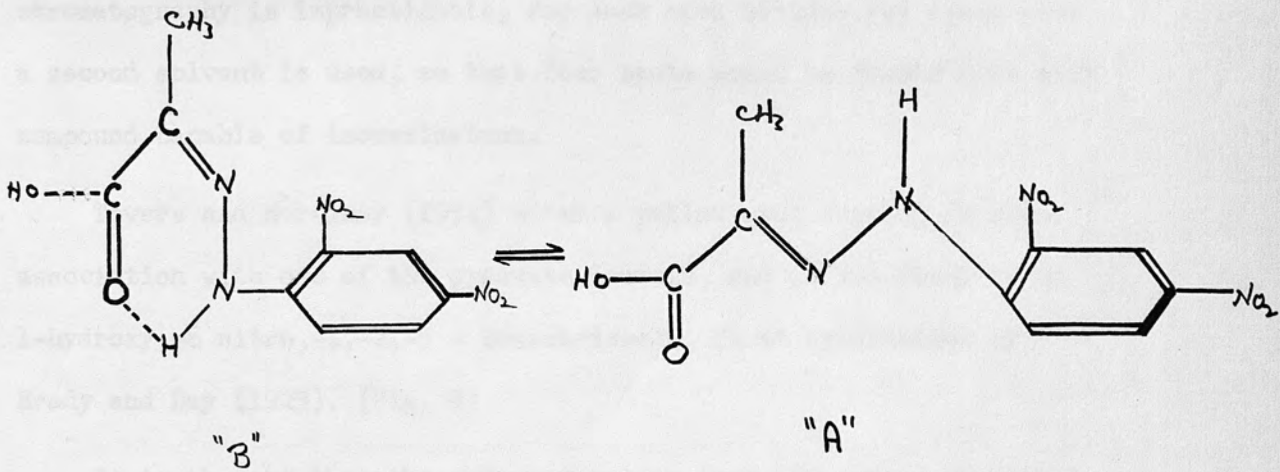


Fig. 7. Isomerisation of pyruvic acid hydrazone.

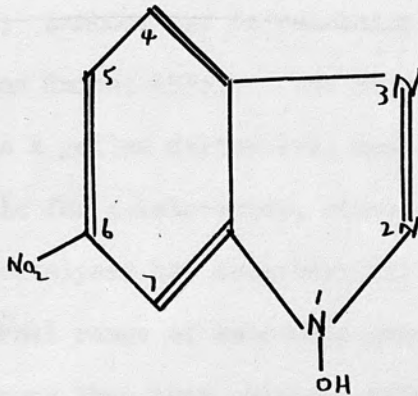


Fig. 8. 1-hydroxy-6nitro,-1-2-3 benzotriazole.

Because of the formation of such isomers, two dimensional chromatography is impracticable, for each spot divides yet again when a second solvent is used, so that four spots would be formed from each compound capable of isomerisation.

Towers and Mortimer (1954) noted a yellow spot running in close association with one of the pyruvate isomers, and it was found to be 1-hydroxy,-6 nitro,-1,-2,-3 - benzotriazole, first synthesised by Brady and Day (1923). (Fig. 8)

It is thought that the compound arises from the action of alkalis on dinitrophenyl-hydrazine; fortunately it remains yellow when NaOH is added, and does not seriously interfere with the colorimeteric estimations of keto-acid hydrazones in alkalis.

5.4. Formation of Nitroquinoxalinols.

In order to avoid the problem of isomerisation of the hydrazones, the use of 1:2 diamino-4-nitrobenzene was introduced (Hockenhull and Floodgate, 1952; Zamboni and Defrancheschi, 1954; Smith and Taylor, 1953; Taylor and Smith, 1955). The compound couples with the keto-acids to produce a yellow derivative, and has the additional advantage of being specific for α-keto-acids, since it does not react with acetone and powerfully catalyses the decarboxylation of β-keto-acids (Edson, 1935). The normal range of keto-acid concentrations in blood is substantially lower than that obtained using 2,4 D.N.P.H., perhaps due to the fact that a solution of the diamino-nitrobenzene reagent autoxidises on standing, to produce a phenazine derivative which rapidly decarboxylates α-keto-acids.

A further disadvantage is that the method is lengthy, requiring a 12-16 hour reaction time, instead of the 30 min. required by the 2,4 D.N.P.H. method.

5.5. Chromatography of Free Keto-Acids.

Chromatography of free keto-acids has been undertaken (Lugg and Overell, 1949; Wieland and Fischer, 1949; Norris and Campbell, 1949; Magasanik and Umbarger, 1950), but the method has not found much favour; experimental conditions are extremely critical, the method is laborious, requiring the use of a locating agent on the developed chromatogram, and only large quantities of keto-acids can be detected. Further, the free keto-acids are relatively unstable. The method appears to be suitable for the analysis of simple mixtures, rather than of the complex mixtures obtained from plant and mammalian sources. (Magasanik and Umbarger, 1950).

6. Column Chromatography.

A few workers have used column chromatography:

Authors	State of keto-acids	Nature of column
Frohman, Orten & Smith (1951)	Free	Silica gel
Le Page (1952)	hydrazone	diatomaceous earth
Datta et al (1950)	hydrazone	alumina
Drew et al (1953)	hydrazone	cellulose powder

These methods are much more tedious than is paper chromatography, and cannot be applied to the analysis of micro quantities. For these reasons most workers have preferred paper chromatography.

7. Miscellaneous Methods of Estimation.

Many specific methods for the estimation of single keto-acids have been introduced, but have rarely been used by any except their originators. They are reviewed by Neish (1957).

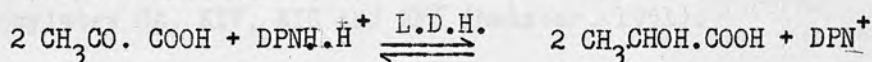
Electrophoresis is not a successful analytic tool (Neish, 1953a, 1953b)

7.1. Enzymatic Methods.

Those with experience of chromatography of keto-acids agree that the method is tedious (Marks, 1961), and in circumstances when only one keto-acid is of interest, a rapid, specific method would be preferable. This need has been partially fulfilled by the introduction of specific enzymatic methods for pyruvate and α-ketoglutarate.

Both are dependent upon the fact that reduced DPN (i.e. DPNH.H*) shows an intense absorption band at 340 mμ, not possessed by DPN. It is thus possible to follow the course of any reaction which requires the co-enzyme, by using ultraviolet spectrophotometry.

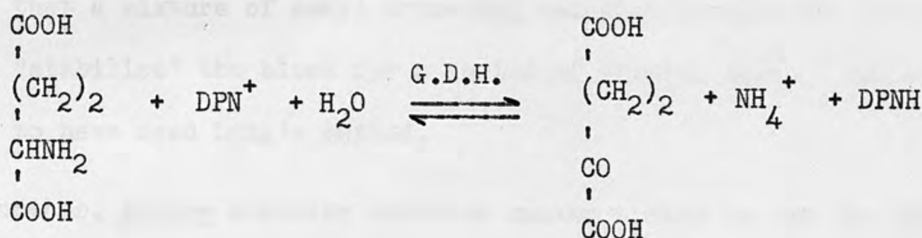
7.1.1. Lactic Dehydrogenase. (Kubowitz and Ott, 1943).



$$2\ CH_3CO.\ COOH + DPNH.H^+ \underset{}{\overset{L.D.H.}{\rightleftharpoons}} 2\ CH_3CHOH.COOH + DPN^+$$

Ochoa et al (1948) believe that LDH is specific but Meister (1950) and Meister and Tice (1950) showed that ketobutyric, hydroxypyruvate, glyoxal and certain α, γ-diketo-acids also acted as hydrogen acceptors though the turnover rates were relatively minute. The method has been applied clinically by Segal, Blair and Wyngaarden (1956) and later by Marks (1961).

7.1.2. Glutamic Dehydrogenase.

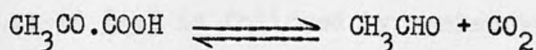


α-Ketoglutarate has been estimated with the enzyme by Seitz, et al (1955); Klingmüller and Vogelsang, (1955); Englhardt-Goelkel et al, (1955); Henley and Pollard, (1955); & Marks, (1961).

Kits of reagents using these enzymes are now available commercially.

7.1.3. Miscellaneous Enzymatic Methods.

Yeast carboxylase is a non-specific enzyme, but, nevertheless, was used by Westerkamp (1933) for the estimation of serum "pyruvate" content.



The evolved CO_2 was measured manometrically, but the enzyme also decarboxylates OA, KIV, KIC and KMV (Meister, 1951).

8. Concentration of keto-acids in human blood.

8.1. Normal subjects.

Before discussing the levels of keto-acids found in normal blood, it is important to consider the methods of collecting specimens.

It has been shown that pyruvate is lost if blood is kept for a few minutes after withdrawal (Bueding and Wortis, 1940). In order to prevent this change, most workers carry out deproteinisation at the bed side, thus immediately inhibiting enzyme action, although Long (1944)

claims that a mixture of cetyl trimethyl ammonium bromide and citrate buffer "stabilise" the blood for a period of several days. Few workers appear to have used Long's method.

Further, severe muscular exercise causes a rise in the pyruvate concentration of venous blood. (Johnson and Edwards, 1937; Lu and Platt, 1939; Friedemann and Barborka, 1941; El Hawary and Thompson, 1953).

On the other hand, moderate activity such as laboratory work does not influence the pyruvate level (Friedemann, Haugen and Kmieciak, 1945).

Lip service has been paid by many to the necessity for avoiding venous stasis and hand clenching when collecting blood samples, but most workers appear to agree that moderate stasis and hand clenching have no effect on the pyruvate concentration in venous blood (e.g. Segal, Blair and Wynngaarden, 1956).

Ingestion of food is followed by a rise in blood "pyruvate" (Friedemann, Haugen and Kmieciak, 1945; Amatuzio, Shrifter, Stutzman and Nesbitt, 1952). Friedemann and Haugen (1943) report two subjects in whom the ingestion of an unstated quantity of glucose caused the blood pyruvate level to rise to 161% and 198% of the fasting level, 2 hours after administration of the carbohydrate.

In order to avoid false levels of keto-acids it is customary to take four precautions:

1) The patient is fasting or should have ingested nothing for 4 hours.

2) The patient should rest for at least ½ an hour before collection.

3) Despite the observations made above, attempts are made to minimise venous stasis during collection.

4) The blood is immediately deproteinised.

Despite these precautions, several workers, including the present author, have noted changes in the blood pyruvate concentration over short periods of time. Thus Segal, Blair and Wyngaarden (1956) noted a falling concentration of pyruvate during hourly sampling throughout the morning, in each of two fasting subjects. The minimal levels reached were 70% and 73% of the respective fasting levels.

They also found considerable variations in any one subject, both from day to day and throughout any one day.

Blood concentration of the keto-acids in resting and fasting or post absorptive subjects, found by various workers, are presented in Table 5.

Several features of the table are of interest.

In those investigations where chromatographic methods were used in parallel with the Friedemann and Haugen method, and where the same initial extraction procedure was used in both, the total keto-acid content measured by the Friedemann and Haugen procedure was greater than the sum of the individual keto acid contents measured by chromatography. (Concentration of <u>all</u> keto-acids being quoted as "pyruvate").

These differences were noted by Cavallini and Frontali (1949a); El Hawary and Thompson (1953) and Bianchessi (1958).

It has been customary to ascribe these differences to the more lengthy procedures involved in chromatography, but it must be noted that McArdle (1957) presented the results of 100 parallel estimations in which the difference between the two methods was by no means great:

TABLE 5 .

Author	Number subjects	Method used	Pyruvate Range mg/ 100 ml.	Pyruvate Mean mg/ 100 ml.	α ketoglutarate Range mg/ 100 ml.	α ketoglutarate Mean mg/ 100 ml.	Branch Chain Range mg/ 100 ml.	Branch Chain Mean mg/ 100 ml.	Comment
Friedemann & Haugen (1943)	4	Own.DNPH derivative	N.Q.	0.79					Total hydrazones 1.29 mg/ 100 ml. (mean value).
Kerppola(1953)	10	Friedemann & Haugen	0.35-0.67	N.Q.					"Pyruvate" is total keto-acids as estim. by Friedemann & Haugen technique.
Frawley (1955)	N.Q.	Friedemann & Haugen	N.Q.	~1.0					Same comment as above. Few details in paper.
Cavallini et al (1949a)	5	Own. Chromatography. of DNPH der.	N.Q.	0.28	N.Q.	0.21			Total hydrazones by F & H method on same blood specs. = 0.88* mg/100 ml.(mean value
El Hawary & Thompson(1952)	15	Own. ditto	0.24-0.69	0.44	0.13-0.24	0.16 (0.08)*			Total hydrazones by F & H = 0.87* mg/100 ml.(mean value)
Seligson & Shapiro(1952)	6	Own.Chromat. of DNPH der.	0.26-0.76	0.54	0.12-0.19	0.15			
Henneman & Bunker(1957)	KG17 P.27	Seligson & Shapiro	N.Q.	1.0 $\pm$ 0.07	N.Q.	0.09 $\pm$ 0.01			
Summerskill et al (1957)	16	Seligson & Shapiro	N.Q.	0.78	N.Q.	0.17			
Hennes et al (1957)	8	Seligson & Shapiro	0.097-0.70	0.31	0.066-0.20	0.091			
McArdle (1957)	8	Own.Chromat. of DNPH der.	0.36-0.81	0.56	0.08-0.18	0.12			Comparison of own method with that of F & H below
Biserte et al (1958)	KG15 P.28	Own.Chromat. of DNPH der.	0.09-0.71	0.39	0.05-0.27	0.13			
De Schepper et al(1958)	?75	Own.Chrom. DNPH der.	0.38-0.90	N.Q.	0.04-0.16	N.Q.	0.43-0.95	N.Q.	Number subjects and means not quoted.
Smith & Taylor (1955)	7	Own Chromat. of DANB der.	0.27-0.47	0.35 $\pm$ 0.07	0.08-0.13	0.10 $\pm$ 0.02			
Segal et al (1956)	14	Enzymatic L.D.H.	0.39-0.86	0.66	-	-			
Hess (1955)	NQ	Enzymatic L.D.H.	N.Q.	0.40	-	-			
Seitz et al (1955)	NQ	Enzymatic glut.dehydrog. on serum	-	-		0.17			
Marks (1961)	20	Enzymatic LDH & glut.dehydrog.	N.Q.	0.462 $\pm$ .147	N.Q.	0.124 $\pm$ .028			

Table5. Blood concentration of keto-acids in normal subjects.

The key to the table is to be seen behind it.

KEY TO TABLE 5

N.Q. = not quoted.

* = as pyruvate.

glut. dehydrog. = glutamic dehydrogenase.

L.D.H. = Lactic dehydrogenase.

Table 5. Blood concentration of keto-acids in normal subjects.

The key to the table is to be seen behind it.

		Mean (mg/100ml)	Range (mg/100 ml)	S.D.
A.	Friedemann and Haugen	1.01	0.42-3.31	0.528
B.	Chromatography	0.97	0.33-3.34	0.542
	Difference (B-A)	-0.04	+0.27 to -0.35	0.102

It will also be noted that the ranges quoted for the various keto-acids vary from author to author, even when the same method has been used.

It is suggested that there are three possible causes for these variations.

1. Small samples. Most workers have studied small numbers of subjects, so that sampling error may be a large factor in the noted variations from author to author.

2. Variation in the state of the subject. It has been noted that activity, ingestion of food, fasting, marked venous stasis and emotional disturbance may cause the pyruvate concentrations to vary. Further, spontaneous variations in pyruvate concentration have been noted to occur in any one subject under constant conditions. It is evident that small variations in the techniques of sampling blood or preparing the subject may cause considerable variation in the results obtained by different workers.

Most of this work, of course, has been directed towards the study of pyruvate alone, either "true" or "total" level being quoted in the literature. "True" pyruvate implies specific estimates of pyruvate by either chromatographic or enzymatic methods, whilst "total" implies the results obtained by procedure A of the Friedemann and Haugen technique (see above, page 24).

3. Accuracy of the methods used. The reliability of the methods used by some workers must remain in doubt, for reproducibility and recovery of added keto-acids are not quoted. (e.g. Henneman and Bunker, 1957; Kerppola, 1953).

8.2 Aceto-acetic acid.

Non-chromatographic methods for measuring blood aceto-acetic acid levels have, with one exception, been non-specific, estimating the "ketone bodies" together (i.e. acetone plus aceto-acetate plus β-hydroxy-butyric acid).

All published methods are based upon the principle that aceto-acetic acid is hydrolysed to acetone by boiling with acid and that β-hydroxy-butyric acid can be oxidised to acetone; acetone and aceto-acetic acid are estimated together, after which the β-hydroxy-butyric is oxidised to acetone and estimated. The acetone resulting from these procedures has been estimated by numerous methods, but the most successful have been to couple it with 2,4 D.N.P.H. (Greenberg and Lester, 1944; Mayes and Robson, 1957; Werk et al, 1955) or with salicylaldehyde (Bakker and White, 1956-57; Thin and Robertson, 1952) and to estimate the product formed colorimetrically.

Thus aceto-acetic acid is usually estimated by difference:

e.g. Aceto-acetic acid = (Acetone + Aceto-acetic acid) - acetone.

or Aceto-acetic acid = Total ketone bodies - (acetone + β hydroxy-butyric acid)

and a true estimate of the aceto-acetic acid content is difficult to obtain; for this reason most authors have preferred to quote the "total ketone body" content of blood (ranging from 0.3 to 1.0 mg/100 ml in normal blood, expressed as acetone).

However, Rosenthal (1949) introduced 4-nitrobenzene diazonium salt as an analytical reagent, the condensation product being measured colorimetrically. This reagent does not react with β-hydroxy-butyrate nor with acetone, but the specificity is of a relatively low order since oxalacetate, pyruvate, tyrosine and thymine all produce coloured complexes, although the molar extinction coefficient of these compounds is considerably lower than that of the aceto-acetate nitrobenzene diazonium complex. In addition Rosenthal found that the aceto-acetate present in protein free filtrates of blood could be easily destroyed by heating in boiling water for 5 minutes, after adding 1/10 volume of 5.0 N.H_2SO_4. He found that pyruvic acid was unaffected by this treatment, and thus aceto-acetic acid was measured by the difference between treated and untreated filtrate.

Rosenthal found a range of 0.095 to 0.26 mg/100 ml. for the blood aceto-acetate concentration in 5 normal subjects.

Thus there is evidence to suggest that aceto-acetic acid is present in normal human blood, although confirmation of the concentrations found by Rosenthal does not appear to have been made.

With these observations in mind, it would seem curious that aceto-acetic acid has been rarely seen in chromatograms of the keto-acid hydrazones. The instability of free aceto-acetic acid, however, has already been noted, and it is probable that the hydrazone of the compound also undergoes decarboxylation during the lengthy extraction procedures, so that the resulting acetone hydrazone is not being extracted into the final alkaline solution. But in conditions where aceto-acetic acid concentrations are high (e.g. in diabetic ketosis),

some of the compound persists until the chromatography step.

El Hawary and Thompson (1953) present evidence for the decarboxylation of aceto-acetic acid during extraction.

9. Urinary Keto-Acids.

Studies on urinary keto-acids have been few, although observations are reported by Biserte and Dassonville (1956), McArdle (1957) and Marks (1961). McArdle noted that the rate of excretion in females is approximately twice that in males, and that it varies with the menstrual cycle, being maximum 2-3 days before menstruation.

Other evidence of the presumed effect of female sex hormones on acids of the citric acid cycle has been presented by Shorr, Bernheim and Taussky (1942) who demonstrated cyclical variations in citrate excretion, the levels being maximal in the middle of the cycle and immediately before the onset of menstruation. The injection of oestradiol into women with amenorrhea increased citrate excretion, although plasma citrate levels remained constant.

In both sexes, the α-ketoglutarate concentration in urine is greatly in excess of that of pyruvate, the reverse of the situation in blood.

Circumstantial evidence exists to suggest that urinary α-ketoglutarate is produced by the kidney cells themselves, in addition to any which may be present in the glomerular filtrate (Krebs, Salvin and Johnson, 1938; Orten and Smith, 1937; Milne, Scribner and Crawford, 1958)

It appears that α-ketoglutarate excretion is related to the state of acid-base balance, for the urinary level in respiratory (but not metabolic) alkalosis increases despite constant blood concentrations; the rise however, is quantitatively small by comparison with the

rise in citrate excretion (Evans, McIntyre,Macpherson and Milne, 1957).

It has been suggested that this change in α-ketoglutarate excretion assists renal compensatory mechanisms in respiratory alkalosis. When human subjects are subjected to prolonged hyperventilation, serum chloride rises whilst HCO_3^- and Na^+ fall, and it is suggested that an increased excretion of α-ketoglutarate and citrate ions conserves the Cl^- which is to be returned to the blood by the kidneys (Cooke et al, 1954; Brown, 1953).

α-Ketoglutarate and pyruvate are the only keto-acids found in measurable quantities in urine, though minute unidentified traces of others have been found occasionally (Biserte and Dassonville, 1956; McArdle, 1957).

10. Keto-acids in Disease.

10.1. Disorders of carbohydrate Metabolism.

10.1.1. Beriberi.

Blood pyruvate concentrations have been used for many years as an index of changes in carbohydrate metabolism, and one of the first to utilise this parameter was Peters, who showed that rats deficient in thiamine had a high blood pyruvate level (Peters and Thompson, 1934; Thompson and Johnson, 1935; Peters, 1936).

Peters suggested that thiamine was essential as a coenzyme in the pyruvic oxidase system. (Fig. 9).

Soon after this, Platt and Lu (1939) showed that the blood pyruvate concentration was raised in <u>acute</u> beriberi, although later workers found that the blood pyruvate level was not often raised in

fasting patients with subacute or chronic beriberi. Administration of glucose to the latter patients did, however, cause a more marked and prolonged rise of the blood pyruvate level than in normal subjects. This test is considered to be more reliable than the simple estimation of fasting blood pyruvate level, in the diagnosis of thiamine deficiency. (Beuding, Stein and Wortis, 1941; Elsom, Lukens, Montgomery and Jones, 1940; Williams, Mason, Power and Wilder, 1943).

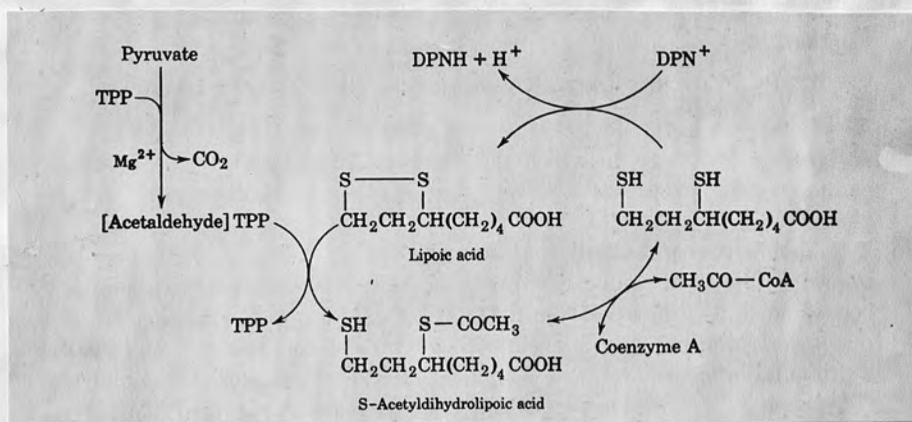


Fig. 9 The pyruvic oxidase system (after Fruton and Simmonds, 1958).

Arsenic, gold, mercury and antimony have been shown to react with the thiol groups of proteins (Thompson and Whittaker, 1947).

Combining this observation with the close similarity in the clinical features of beriberi neuropathy and arsenical poisoning, Joiner, McArdle and Thompson (1950) were led to investigate the effect of thiamine on patients with neuropathy of no known aetiology.

The patients were given glucose, and those showing an exaggerated blood pyruvate rise were treated with massive doses of thiamine, the test being repeated after this. If the patient showed no change in his response to glucose, he was given dimercaprol (BAL) on the assumption that some heavy metal was responsible for the neuropathy. The results were disappointing.

"Epidemic dropsy" occurred in widely scattered districts of India, and intensive epidemiological study of the disease (Lal et al, 1939; 1940; 1941) led to the conclusion that the victims had ingested mustard seed oil contaminated with oil expressed from the seeds of the plant <u>argemone mexicana</u>. Subsequently Sarkar (1948) isolated the toxic alkaloid sanguinarine, which he showed, by <u>in vitro</u> studies, to inhibit the pyruvic oxidase system and to cause a rise in the concentration of pyruvate in rat blood. This finding agrees with the observations of Wilson and Ghosh (1937) that the bisulphite binding capacity of the blood rose in cases of epidemic dropsy, although no one appears to have measured the pyruvate concentration in this condition.

Sarkar (1948) drew the conclusion that interaction between the alkaloid and the -SH groups of pyruvic oxidase occurs, to inactivate the enzyme.

10.1.2. <u>Diabetes Mellitus.</u>

In view of the widespread nature of the disease, it is not surprising that much work has been undertaken in an attempt to obtain an understanding of the biochemical lesions responsible for diabetes mellitus.

Unfortunately, as in many other fields in which keto-acids have been studied, the results have been conflicting, some authors finding

high values, others normal, e.g.

Author	Findings (Blood)
Villano (1952)) Seligson & Shapiro (1952))	α-Ketoglutarate and pyruvate raised in fasting diabetics.
Bending, Wortis, Stern &) Esturonne, (1942)) Klein (1942))	Pyruvate normal in fasting diabetics.
Lestradet and Guest (1951)) Von Marken Lichtenbeldt and) Florijn (1952(1955))	Normal blood pyruvate in diabetic ketosis.

Similarly, the response to administered glucose was found to vary. Many have reported no rise in blood pyruvate, though all normal subjects showed a rise after glucose administration (Klein, 1942; Bending, Wortis Fein and Esturonne, 1942; Miller et al 1952; Himwich and Himwich, 1946)

However, Root, Stotz and Carpenter (1946) and Amatuzio et al (1952) found that patients with mild disease responded to glucose administration with an elevation of blood pyruvate, whilst those with severe disease showed no response. These findings are compatible with the existence of two types of diabetics: those requiring insulin, with no insulin-like activity in the plasma, and those not requiring insulin, with demonstrable plasma insulin-like activity. (Lawrence, 1951; Bornstein, 1950; Vallance-Owen et al, 1955).

This problem was further explored by Smith and Taylor (1956) using paper chromatography of the nitroquinoxaline derivative of the keto-acids. These authors found no rise in the pyruvate level in insulin requiring diabetics (after discontinuing insulin therapy for 12 hours), though those not requiring insulin showed a response after administering glucose.

α-Ketoglutarate was not significantly altered during the test, and fasting levels of α-ketoglutarate and pyruvate were found to be within normal limits. This group have confirmed and extended these observations. (Anderson, Marks and Smith, 1961; Anderson and Marks, 1962). Their results confirmed the observations of Smith and Taylor, and in addition they found that fasting, insulin requiring diabetics given 5 units of crystalline insulin intravenously at the same time as they were given 100 g. of glucose orally showed a much greater rise in the blood pyruvate concentration than did either normal subjects or obese, non-insulin requiring diabetics treated similarly.

The authors explain the last observations by writing "When glycolysis proceeds normally in insulin requiring diabetics, some of the cells apparently have an altered permeability to pyruvate, which therefore diffuse rapidly into the blood."

Moorehouse (1959) has shown that insulin requiring diabetics are unable to deal with an intravenous load as rapidly as healthy subjects; insulin therapy, achieving excellent clinical control of diabetics did not abolish the abnormality, although it was sometimes reduced in degree.

Anderson and Marks (1962) and Takanami et al (1960) found that insulin requiring diabetics deprived of the hormone had an abnormally high urinary pyruvate excretion , both in the fasting state and after ingesting glucose. The pyruvate concentration in blood was normal, and the authors suggest that the kidneys of these patients cannot reabsorb pyruvate as efficiently as healthy subjects or obese diabetics, both of which groups showing very low rates of pyruvate excretion.

In the insulin requiring diabetics, urinary pyruvate was not significantly diminished when insulin was given with oral glucose, but since the pyruvate load delivered to the kidney was more than doubled, they reasoned that renal pyruvate conservation improved somewhat. The rates of urine flow and urinary pH were similar in all three groups of patients, so that the authors excluded a non specific osmotic diuresis or pH effect as an explanation of the excessive pyruvate excretion.

10.1 3. The Adrenal Glands and Keto-acids.

Evidence that blood pyruvate concentration was raised in pulmonary oedema, myocardial infarction, peripheral embolism, hypertensive encephalopathy, cerebral haemorrhage, pneumonia, biliary colic and haematemesis (Kleeberg and Gitelson, 1952; 1953; Gitelson and Tiberin, 1952) led Gitelson (1953) to conclude that the rise might be due to increased adenocortical activity in the stress reaction. He studied the blood pyruvate levels in patients given A.C.T.H. or cortisone:

a) Acute experiment (1 dose of drug)

Drug + route of administration.	No. of subjects	No. of subjects showing rise in pyruvate conc.	No. of subjects showing fall in pyruvate conc.
25 i.u. A.C.T.H. I.M.	21	21	-
100 mg. cortisone, I.M.	8	5	3

B) Chronic experiment. Twenty patients given the same dosage of drugs as above daily for 2-3 weeks, all showed significant rises in pyruvate.

Kerppola (1953) was fortunate enough to follow the development of Cushing's Syndrome in three patients and presented his results for blood pyruvate:

0.87 mg/100 ml. - 3 months later - 1.20 mg/100 ml.
0.53 mg/100 ml. - 2 months later - 0.78 mg/100 ml.
1.25 mg/100 ml. - 3 months later - 1.58 mg/100 ml.

He found a <u>normal</u> range of 0.35 - 0.67 mg/100 ml.

It is interesting that he found two cases of Addison's Disease to have levels of 0.35 and 0. 63 mg/100 ml. respectively. He found a rise of blood pyruvate of from 10 to 100% in 17 of 23 patients given hydrocortisone over a prolonged period.

Hennemann and Bunker (1957) reported very high pyruvate levels in 13 patients with Cushing's Syndrome or who had been given long-term cortisone therapy; in two of three Cushing patients submitted to adrenalectomy, the postoperative level of pyruvate was lower than the preoperative. (One patient died at operation). These workers noted α-ketoglutarate levels no different from normal.

Hennes, Wajchenberg, Fajans and Conn (1957) found that 10 patients given prednisolone all had abnormally high pyruvate levels.

Whilst it would appear that enhanced adrenocortical activity may cause a rise in blood pyruvate, it is important to recognise that not all patients in this state have high blood pyruvate concentrations. Thus Segal, Blair and Wyngaarden (1956) reported two patients with Cushing's Syndrome who had normal levels.

There seems to be no consistent relationship between high blood levels of pyruvate and a diabetic type of glucose tolerance curve.

This is illustrated by the results of Hills, Power and Wilder (1952) on 11 patients with Cushing's Syndrome.

No. of patients	Diabetic glucose tolerance curve	Raised fasting blood pyruvate
4	Yes	Yes
5	No	No
2	Yes	No

Hennemann and Bunker (1957),however, claim that a raised fasting pyruvate level precedes the appearance of an abnormal glucose tolerance curve, as the severity of Cushing's Syndrome increases.

10.2 Liver Disease

Many workers have reported raised blood levels of keto-acids in various liver diseases, though they have not been consistently raised in all patients, and a few results have been in conflict.

Bianchessi(1958) has reported that blood pyruvate, measured by the Friedemann and Haugen and chromatographic methods, was raised in hepatic coma, but not in "compensated" liver disease, whilst Amatuzio and Nesbitt (1950) reported normal levels in viral hepatitis, but "mild" elevation in compensated cirrhosis; in hepatic coma the level was high and fluctuated with the clinical state. Unfortunately the last two authors did not quote actual values.

Seligson (1952), using his own chromatographic method, reported elevated fasting levels in some patients:

18 normal controls yielded: α-ketoglutarate level 13.0 ± 1.5μ M/litre

pyruvate level 73.0 ± 16 μ M/litre

Diseased patients revealed the results below:

Disease	No. of patients	Number of patients in whom KG level > Mean + 2 S.D.	Number of patients in whom Pyruvate level > Mean + 2 S.D.
Acute viral hepatitis	18	16	5
Chronic hepatitis	16	13	5
Cirrhosis	19	16	11

This work was followed by a well controlled investigation of blood keto-acids in 27 patients with hepatic cirrhosis by Summerskill, Wolfe and Davidson (1957), using a chromatographic method of study:

Condition	Mean Levels.	
	α-ketoglutarate (μM/litre)	Pyruvate(μM/litre)
Controls	11.5	89
"Compensated" cirrhosis	20.7	112
Impending coma	32.3	154
Coma	39.7	225

They found, however, that the level of α-ketoglutarate in ⅓ of patients in impending coma or coma and ½ of the pyruvate levels in both these states lay within the upper limits for the values in "compensated" cirrhosis. These authors found that α-ketoglutarate, and usually the pyruvate, rose and fell with a worsening or improvement of the patients mental state; although the latter level occasionally continued to rise, even when the patient improved.

The authors suggest that there is some block in the utilisation of pyruvate in cirrhosis and in coma, supporting the suggestion of Amatuzio, Shrifter and Stützman and Nesbitt, S., (1952) who noted a prolonged rise in pyruvate after administration of intravenous glucose to patients in coma, though normal subjects or those with compensated cirrhosis did not show this phenomenon.

The significance of raised blood ammonia in hepatic coma is obscure, and the level seems to have little prognostic value. Nevertheless, the observations of Recknagel and Potter (1951) that when ammonia is added to rat liver breis or homogenates, the citric acid cycle is blocked and acetoacetate is formed, led them to postulate that the ammonia combined with α-ketoglutarate to form glutamic acid, thus reducing the concentration of an essential substrate for the efficient functioning of the citric acid cycle.

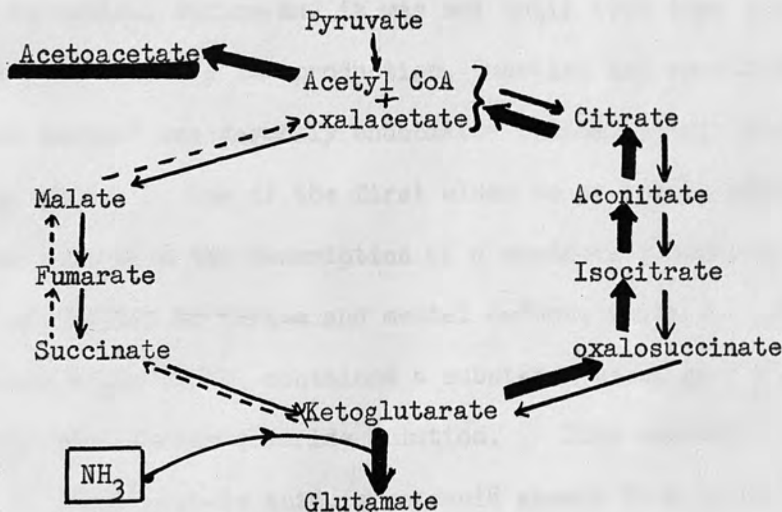


Fig. 10. Effect of NH_3 on Krebs Cycle.
Heavily printed arrows represent effect of NH_3.

This effect of ammonia has been postulated as the mechanism responsible for the cerebral symptoms in hepatic coma, amination of the α-ketoglutarate rapidly reducing the small quantity of the substance present in brain. Replacement of the α-ketoglutarate from the blood would be very slow because of the considerable blood-brain barrier for this compound which exists (Bessman, 1959; Eiseman, 1957; McArdle, 1957).

Some doubt is thrown upon this theory by the fact that the severity of the neuropsychiatric symptoms cannot be correlated with the blood ammonia concentration (Fisher and Faloon, 1956).

10. 3 Genetics and Keto-acids.

"Inborn errors of metabolism" and the principles of congenital enzyme deficiencies were first enunciated by Garrod in his Croonian Lectures (1909), but for many years the disorders he described were regarded as medical curios, and it was not until 1959 that the theory that "one gene controls the production, function and specificity of a particular enzyme" was formally enunciated (Tatum, 1958; Beadle, 1958; Lederberg, 1959). One of the first clues to an inborn error of metabolism came with the description of a syndrome occurring in infancy, of failure to thrive and mental defect, while the urine, some 6 to 8 weeks after birth, contained a substance which gave a greenish-blue colour with ferric chloride solution. This substance was later found to be phenylpyruvic acid, a ketoacid absent from normal urine. (Fölling, 1934).

Later work showed that the livers of these children lacked phenylalanine hydroxylase (Jarvis, 1947; Udenfriend and Bessman, 1953)

and phenylalanine was found to accumulate in the blood (Armstrong and Bentley, 1956).

Meister, Udenfriend and Bessman (1956, 1958) demonstrated an increase in phenylalanine transaminase activity, and suggested that the increase was induced by a high level of phenylalanine; the delay in the appearance of phenylpyruvic acid in the urine was ascribed by Knox and Hsia (1957) to the time required to induce the increased enzyme activity.

Phenylpyruvic acid is also decarboxylated to form phenylacetic acid which is conjugated and excreted as phenylacetyl glutamine (Meister, 1958, Meister, Udenfriend and Bessman, 1956).

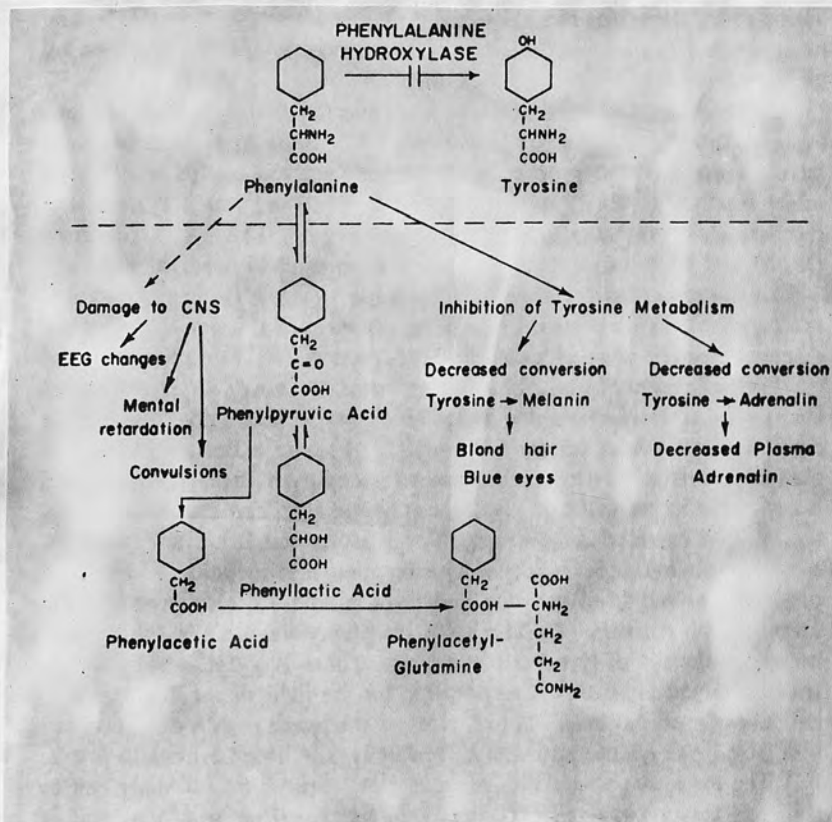


Fig. 11. Schematic representation of the biochemical lesion in phenyl ketonuria. (After Hsia, 1960).

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Early recognition of this condition is of great importance, since feeding the child on a diet free of phenylalanine results in an apparently normal development (Hsia, 1960).

A further syndrome of severe mental retardation, failure to thrive and a rapid downhill course to a fatal termination some 18 days to 20 months after birth has recently been described, and is associated with a urine smelling of maple syrup.

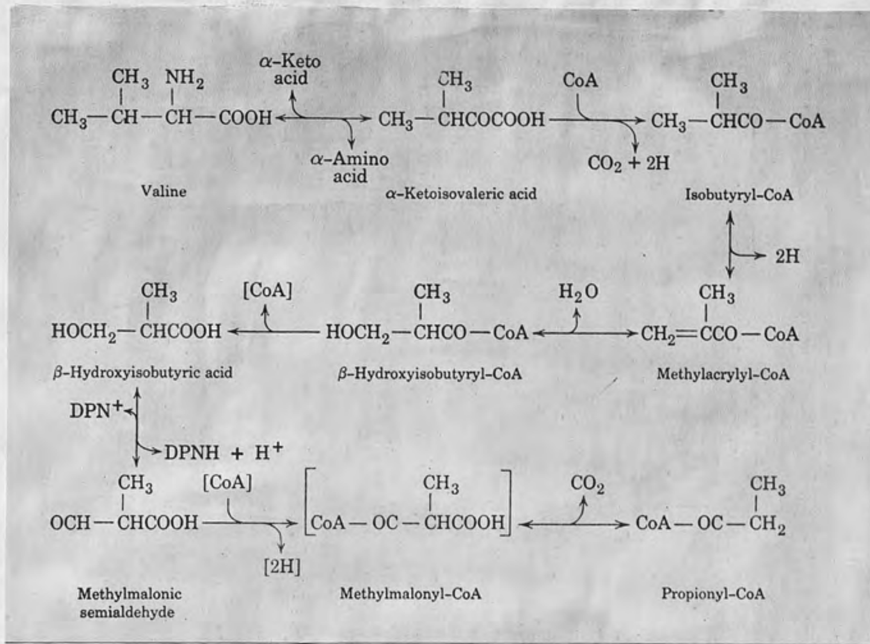
Some 13 cases have now been reported (Menkes, Hurst and Craig, 1954; Westall, Dancis and Miller, 1957; Smith and Strang, 1958; MacKenzie and Woolf, 1959; Menkes, 1959; Dancis, Levitz, Miller and Westall, 1959).

Westall et al (1958) found a raised level of the "branch chain" amino acids leucine, isoleucine and valine in both blood and urine, and MacKenzie and Woolf (1959) found high concentrations of the ketoacids corresponding to these amino acids in the urine.

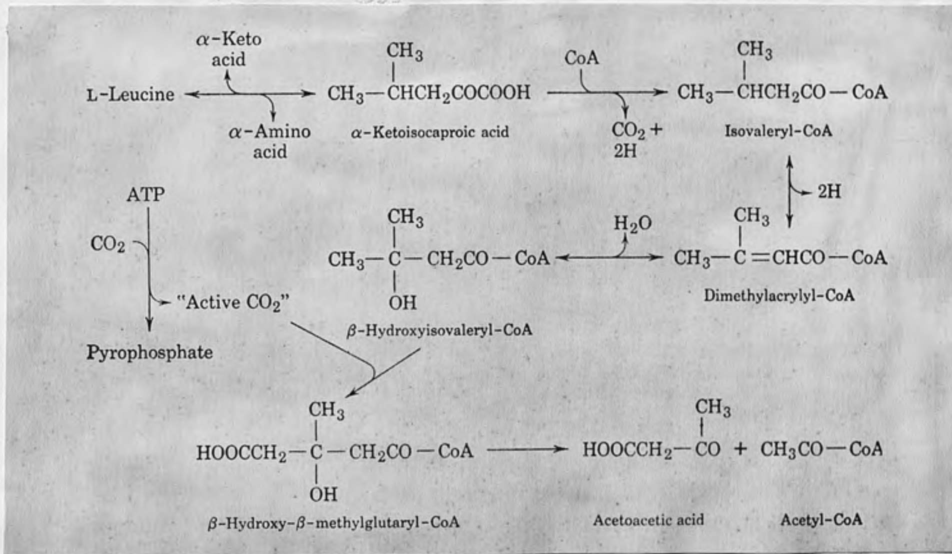
The precise nature of the biochemical lesion in this disease has not been defined. Transaminase activity for the branch-chain amino acids was found in the tissues of a child with the disease (Westall, Dancis, Miller and Levitz, 1958; Westall, Dancis and Miller, 1957; Menkes, 1959; Dancis et al, 1959).

Menkes (1959) proposed a block at some point on the catabolic pathway of the branch-chain ketoacids, and it was suggested that its site was at the stage of oxidative decarboxylation of the keto-acids (MacKenzie and Woolf, 1959; Menkes, 1959).

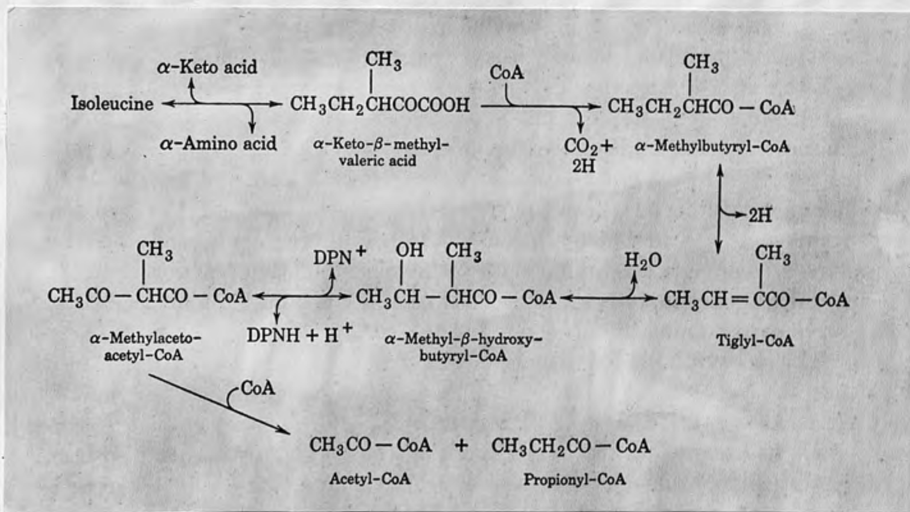
Unfortunately attempts by Dancis et al (1959) failed to pinpoint the lesion, since control tissue from a child who died of trauma failed



Valine



Leucine



Isoleucine

Fig. I2 The catabolism of branch chain amino acids (Fruton & Simmonds, I958)

to metabolise added leucine -1- ^{14}C.

Figure 12 presents the normal catabolic pathway of leucine, iso-leucine and valine, and it is evident that the absence of an enzyme catalysing any one step common to the degradative pathway of all three, would cause an accumulation of the amino acids, and also of the corresponding α-ketoacid.

Unfortunately none of the enzymes catalysing these steps has been isolated. Until this is done, and their specificity defined, the precise biochemical lesion in this syndrome must remain in doubt.

11. In Summary.

From the short survey presented above, it is apparent that the keto acids are intimately involved in many metabolic processes, and that the potential field of study, both in health and disease, is extensive.

From the beginning, it was obvious that it would be necessary to limit the field of study, in order to obtain any useful results.

Part II, below, describes a study of methods, with a detailed description of the method eventually chosen, and Part III describes the results obtained by applying it to selected problems.

Part 2. Methods.

PART II.

METHODS.

As has already been noted, there is no ideal method for estimating all the known ketoacids present in blood at the same time. The method of De Schepper et al (1958) is the most comprehensive to date, the hydrazones of five ketoacids being present in one chromatogram, although the "branch chain" compounds all travel in one composite spot. It was decided to investigate methods further, in the hope of separating and estimating all the individual ketoacids present in blood:

1. Free ketoacids.

Magasanik and Umbarger (1950) had successfully separated α-ketoisovalerate and α-keto-β-methylvalerate when the two compounds were chromatographed as their free acids. Unfortunately these authors had not studied the chromatographic behaviour of all the ketoacids which are of interest here. Accordingly it was decided to attempt the separation of various ketoacids using essentially their method. Complete experimental details may be found by referring to the relevant papers (Magasanik and Umbarger, 1950; Umbarger and Magasanik, 1952) and only the outline is described below:

After deproteinisation of the tissue of origin with the tungstic acid precipitant of Folin and Wu, suitable aliquots of some 10-50 μL were spotted onto Eaton and Dikeman 613 chromatography paper, and ascending chromatography, using one of two solvent systems, was carried out:

Solvent system 1 = n-Butanol: formic acid = 95:5

Solvent system 2 = S-Butanol: propionic acid = 95:5

saturate both mixtures with distilled water.

After drying the developed chromatograms, the paper was heavily sprayed with an aqueous solution containing 0.1% of semicarbazide hydrochloride and 0.15% of sodium acetate. After drying, the paper was viewed by transmitted U.V. light when the opaque spot of ketoacid semicarbazide could be identified against a fluorescent background. The spot was outlined with pencil, cut from the paper and mixed with a dilute solution of 2,4-dinitrophenylhydrazine. Eventually the characteristic pink colour of the ketoacid hydrazone was developed with KOH and the compound determined colorimetrically.

The following minor modifications of this procedure were adopted.

1. Although the original workers found that only E. and D. 613 paper was sufficiently inert to semicarbazide, the present author found that no false U.V. opaque spots were produced on Whatman No. 1 or No. 4 paper. Because E. & D. 613 was not readily available, the Whatman papers were used, particularly No. 1, since it had the speed characteristics of the paper used by Magasanik and Umbarger.

2. Three solvent systems were used:

   1. n-Butanol: formic acid = 95:5
   2. sec-Butanol: propionic acid = 95:5
   3. n-Butanol: propionic acid = 95:5

   (All three were saturated with distilled H_2O).

Aqueous solutions of free ketoacids whose preparation is described below, were made up in 0.1% (w/v) solution so that 20 mg. could be spotted onto the paper in a convenient volume.

Initial experiments were undertaken using free pyruvate and

α-ketoglutarate, and it was found that the two compounds travelled with slightly differing speeds, but streaking was marked so that it proved impossible to resolve a mixture of the two during a reasonable length of solvent run.

The above observation, combined with the disadvantages listed below led the author to believe that the method was unsuitable for the project in hand:

1. In order to put a sufficiently large load of ketoacid onto the paper, particularly in the case of α-ketoglutarate, it was necessary to spot the paper with large volumes of deproteinised blood extract (Magasanik and Umbarger found a limit of sensitivity of 5 µg) and 6 ml. of the extract woŭld be required to achieve a 5 µg load for pyruvate.

2. The relative complexity of the method, with two separate drying operations and with spraying, added further to its tedious nature, rendering the process ill suited for application to clinical application.

3. The solvent systems were extremely sensitive to temperature change, and, despite stringently carrying out the detailed instructions of Magasanik and Umbarger in their preparations, a fall of only a few ^{o}C in the atmospheric temperature resulted in separation of the phases.

4. Streaking. Before finally abandoning the method, attempts were made to eliminate streaking:

a) small variations in the acid content of the systems had no beneficial effect.

b) Careful equilibration for 24 hours with damp paper lining the tank wall had no effect.

c) Substituting the slower No. 1 for No. 4 paper had little effect.

5. Oxalacetate. An interesting finding was that large amounts (50 μg) of oxalacetate spotted onto the paper produced a U.V. opaque spot when sprayed with semicarbazide immediately, but subjecting the substance to chromatography first made it impossible to locate the spot on subsequent spraying.

The instability of oxalacetate has been mentioned in part I, but decarboxylation with formation of pyruvate during development would leave behind a residue still capable of reacting with semicarbazide; presumably decomposition during this chromatographic procedure produces some substance other than pyruvate.

Despite the excellent recoveries and reproducibility reported by the authors, it was felt that the above preliminary findings were sufficient to make the method unsatisfactory for the present study; it must be pointed out that they found it of great value for the study of relatively simple mixtures in the culture media of micro-organisms.

2. Ketoacid hydrazones.

After the experience described above, it was decided to return to the old established method of studying the hydrazone derivatives, and to attempt to evolve a method which would separate the derivatives of all the ketoacids known to be present in blood.

2.1. Separation of Synthetic hydrazones by Chromatography.

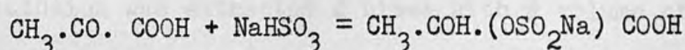
2.1. 1. Material and Methods.

(a) Pyruvate Hydrazone. Commercially available pyruvic acid is said to contain up to 30% of γ-valerolactone (Clift, and Cook, 1932) and it is necessary to use freshly distilled pyruvic acid to obtain its pure hydrazone.

Commercial grade pyruvic acid (British Drug Houses, Ltd) was twice redistilled at 35 mm. Hg. pressure, the yield being approximately 50% of the original yellow liquid; the distillate was colourless, leaving a dark brown viscous residue, presumably mainly γ-valerolactone.

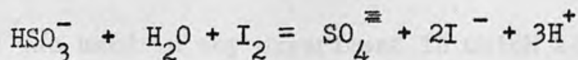
The distillate was dissolved in water to make a 20% (w/v) solution and neutralised by the addition of 90% of its equivalent weight of solid, "Analar" sodium bicarbonate. Six volumes of acetone were added to precipitate sodium pyruvate, and the whole was kept in the refrigerator overnight. The pyruvate was filtered off, dissolved in a minimum of water, and treated with acetone until strong turbidity occurred. The mixture was then warmed until the pyruvate dissolved, and cooled in ice and salt mixture. The sodium salt was filtered off and dried over calcium chloride at 50°C until its weight was constant.

(b) Purity of Pyruvate. The method of Clift and Cook (1932) was used for estimating the purity of the pyruvate. This is dependent upon the bisulphite reaction.



$$CH_3.CO.\ COOH + NaHSO_3 = CH_3.COH.(OSO_2Na)\ COOH$$

The excess sodium bisulphite is titrated against iodine:



$$HSO_3^- + H_2O + I_2 = SO_4^{=} + 2I^- + 3H^+$$

The bisulphite- pyruvate complex is split by the addition of saturated $NaH.CO_3$ solution and the bisulphite equivalent to pyruvate thus estimated.

After preliminary standardisation of N/10 iodine solution against thiosulphate, and of bisulphite solution against iodine, the purity of the sodium pyruvate was found to be 93%.

(c) Preparation of Pyruvic Hydrazone. 0.2 g. of sodium pyruvate was dissolved in 20 ml. water and a slight excess of 0.1% 2,4-

dinitrophenylhydrazine in 2N.HCl added. A crop of crystals was thrown down immediately and was filtered off.

The supernatant was left overnight in the refrigerator and a further crop of crystals removed the next morning.

The two crops were combined, dried overnight over $CaCl_2$ and recrystalised from glacial acetic acid.

(d) <u>Ketoglutarate Hydrazone.</u> Fresh α-ketoglutaric acid (M.P. 110°C., uncorrected; from L. Light and Co., Colnbrook) was used, the details being as for pyruvate.

The dry hydrazone was recrystallised from cold ethyl acetate using petroleum ether (60-80°B.P.)

(e) <u>Acetoacetate Hydrazone.</u> Acetoacetic acid was prepared as its sodium salt by the method of Shaffer (1921):

Ethyl acetoacetate was saponified with 2N.NaOH overnight at 37°C. The solution was cooled and acidified to congo red paper with H_2SO_4.

The solution was extracted 4 times with ½ volume of ether in a separating funnel, and the ether layer then shaken with water made slightly alkaline with NaOH. This alkaline solution contains the sodium acetoacetate and was used in any experiment in which acetoacetate was required, after passing N_2 through the solution to remove acetone formed by spontaneous decomposition of the β-ketoacid.

The filtered solution was kept in the refrigerator.

(f) <u>Ketoisovalerate Hydrazone:</u> KIV was obtained from the Sigma Chemical Corporation, St. Louis, Mo, and the hydrazone prepared as for pyruvate, recrystallisation being undertaken from ethyl acetate, as in the case of oxalacetate.

(g) Ketoisocaproate. The free acid was obtained as a liquid from the Californian Corporation for Biochemical Research.

The hydrazone was prepared in the usual way and the resulting compound recrystallised from hot water.

(h) α-keto-β-methylvaleric hydrazone. Only a small quantity of KMV was available, generously donated by Professor Alton Meister of Tufts University School of Medicine, Boston, Mass.

5 mg. of the sodium salt was dissolved in 10 ml. H_2O and the exact equivalent of the D.N.P.H. solution added.

The precipitate was filtered off and dried, but it was not recrystallised.

(i) Acetone Hydrazone. This was prepared in the usual way from "Analar" acetone in a 5% (v/v) aqueous solution and was recrystallised from glacial acetic acid.

Purity of the Hydrazones.

For the purpose of this work, M.P. determinations were regarded as satisfactory criteria of purity, since the elemental composition of some of the hydrazones resemble each other closely; indeed, those of KMV and KIC are merely isomers of each other.

Keto-acid	Found M.P., °C. (Uncorrected)	Comments	M.P. quoted °C	Reference
Pyruvate	216	Sharp M.P.	218	1
α-ketoglutarate	219	"	219	1
Oxalacetate	211	"	211	1
α-ketoisovalerate	194	-	196	2
α-keto-β-methylvalarate	166	Sharp M.P.	168	2
α-ketosiocaproate	159	-	160	2
Acetone	126	Sharp M.P.	126	1

Reference 1 = El Hawary + Thompson, 1953.
Reference 2 = Meister + Abendshein, 1956.

It will also be noted that the standard of purity of only pyruvic acid was determined (see above). This was necessary because of the high instability of the compound; the other α-ketoacids are more stable, and the M.P. of the hydrazone was regarded as adequate proof of purity.

2.1. 2. <u>Chromatographic Separation of the synthetic hydrazones.</u>

Reference to the published methods for chromatography of ketoacid hydrazones revealed that α-ketoglutarate, with a very low Rf value, is well separated from the pyruvic acid, and both are separated from the "branch chain" ketoacids which travel together in a composite spot (De Schepper et al, 1958).

The first studies undertaken, therefore, were on the chromatographic behaviour of the branchchain ketoacids, with a view to possible separation.

a) <u>Conventional solvent systems.</u> Saturated solutions of the hydrazones prepared above were made in distilled water and were renewed at frequent intervals.

Usually 20 µl of each solution were spotted onto the chromatography paper using a micro-pipette and dried by a stream of compressed air, at room temperature. to dry the spots.

Whatman 3 MM paper was used in this study. Altmann et al (1951) and De Schepper, Parmentier&Van der Haeghe (1958) found that the spots were more compact on buffered paper; accordingly the strips were dipped in 0.1 M Na barbitone - HCl buffer of pH values, 6,7,8,9 and 10, and then blotted dry; they were allowed to dry at room temperature. Unbuffered papers were used, also.

The hydrazone solutions were spotted at the origin(which was 10 cm. from the narrow end of a 10 x 40 cm. strip of paper) and each was 2 mm. in diameter and 2.5 cm. from its neighbour.

After overnight equilibration with the vapour of the aqueous phase of the solvent in a sealed glass tank the organic phase was introduced, and descending development carried out for 12 hours.

Three solvent systems were used:

1. n-Butanol: ethanol:water = 5: 1: 4 (Meister, 1956).
2. n-Butanol: ethanol: 0.5N. NH_4OH = 7: 1: 2 (El Hawary, 1953).
3. Isoamylalcohol: ethanol: H_2O = 5: 1: 4 (Altmann et al, 1951).

In the case of all three, although α-ketoglutarate and the isomers of pyruvate hydrazones were well separated from each other and from the "branch chain" derivatives, these last three were not resolved.

This experience was, apparently, shared by De Schepper et al (1958) and by Meister and Abendschein (1956) who studied 14 solvent systems, eight of which were found unsatisfactory for some technical reason, and the remaining six failed to separate the branch chain derivatives.

It was therefore decided to investigate some less conventional systems.

b) Acidic Solvent Systems.

(I) Theoretical Considerations.

In a theoretical treatment of partition chromatography, Martin(I959) showed that:

$$\alpha = \frac{Al}{As} \times \left[\frac{I}{Rf} - I \right] \text{ - - - - - - - - - Equation I.}$$

where α is the partition coefficient of solute between water and solvent phases.

Al is the cross sectional area of the solvent phase

As is the " " " " " water "

and $Rf = \dfrac{\text{Movement of band of solute}}{\text{Movement of solvent front}}$

From equation I,

$$\ln \alpha = \ln \frac{Al}{As} + \ln\left(\frac{I}{Rf} - I\right] \text{ - - - - - - - - - Equation 2}$$

But for a given solvent system, paper and temperature $\frac{Al}{As}$ is constant, and therefore

$$\ln \alpha = K + \ln \left[\frac{I}{Rf} - I\right] \text{ - - - - - - - - - Equation 3}$$

Bate-Smith and Westall(I950) called the variable quantity on the right hand side of equation 3 the "Rm value", i.e.

$$Rm = \ln\left[\frac{I}{Rf} - I\right] \text{ - - - - - - - - - - - - - Equation 4}$$

Further thermodynamic considerations led Martin(I959) to conclude that the Rm value is actually the sum of similar values for the individual component groups of the whole molecule, e.g.

$$R_m \text{ glycine} = \Delta R_m NH_2 + \Delta R_m CH_2 + \Delta R_m COOH \text{ - - - -Equation 5}$$

These theoretical considerations have received experimental

verification from Bremner(I95I) and Serchi(I953) for amines,from Bate-Smith and Westall(I950) for plant pigments and from Bush(I960) for steroids.

Consideration of equation 4 shows that Rm and Rf are closely related and that knowledge of Rm values of certain molecules in specific solvents should allow a prediction of Rf values to be made,where ideal solutions are dealt with.

The solvent systems used in the chromatography of keto-acid hydrazones have been universally neutral or alkaline,with the exception of that of Wallgren and Nordlund(I956),containing I0% acetic acid in the mobile phase:

Non-Acidic Solvent Systems.

Solvent(composition by volume)	Reference
Tert-amyl alcohol:etOH;water 5:I:4	Altmann,et al(I95I)
n-butanol:etOH:0.5N.ammonia 7:I:2	El Hawary&Thompson,(I953)
n- butanol:I.0N. $NaHCO_3$ I:2	Seligson&Shapiro(I952)
glycine-NaOH buffer (0.IM),pH 8.4	Virtanen,et al (I953)
n-butanol:water:etOH 5:4:I	MEISTER(I956)
methanol:benzene:n-butanol:water 4:2:2;2.	Meister and Abendschein(I956)

Wallgren and Nordlund found that their acidic solvent system was unstable,and others have had similar experience with this system. Thus Bate-Smith and Westall(I950) found that the slow esterification of butanol caused changes in Rf values of plant pigments,when all other conditions remained constant.

Magasanik and Umbarger (I950) tried to avoid these changes by allowing their solvent systems containing butanol and formic or propionic acid to "age" for a month before use.Nevertheless,these workers found their solvents to be unstable,as did the present author.

In studies of the effect of solvent composition on hydrazone separation,Bush and Hockaday(I960) compared three acidic solvent systems with each other and with the butanol:3% aqueous NH_3 system used by Cavallini and Frontali(I949).

The three systems contained hydrocarbons.(I960)

The three hydrocarbon systems of Bush(I960)

Code Name	Composition(by volume)
T/A75	Toluene/acetic acid/water 4:3:I
LTII/A85	Light petroleum*/toluene/acetic acid/water IO:IO:I7:3
D/A 90	Decahydronaphthalene/acetic acid/water IO:9:I

*- fraction boiling at IOO-I20°C.

In their rather brief report,Bush and Hockaday(I960) pointed out that;

I. Δ Rm values decreased with increasing chain length of the chain length of the hydrazone,but

2. The Δ Rm value is greater in the acid systems than in the alkaline system they studied.

Thus there seemed to be a reasonable prospect of separating all the major hydrazones completely,using thes solvent systems,although Bush and Hockaday merely reported the chromatographic behaviour of the α-ketoglutarate and pyruvate derivatives;they claimed that the latter did not form two separate isomeric spots,but that it merely formed one slightly elongated spot.

Hockaday(I96I) reported that the hydrazones decomposed in the acidic conditions,but that the percentage loss was directly proportional to the load on the paper.

(2) Experimental.

Materials

I. Ligroin(light petroleum,fraction boiling IOO-I20°C,M.andB. Chemicals)
2. Decahydronaphthalene ("Decalin",B.D.H)
3. Toluene(sulphur free,B.D.H.)
4. Acetic acid(Reagent grade,M.andB.)

Chromatography paper

IO cm. by 40cm.strips of Whatman paper,nos.I,4 and 3M.M. were prepared.

Solvent systems.

The three solvent mixtures of Bush were prepared,and allowed to stand at room temperature for I week before use.

Standard hydrazone solutions

Aqueous solutions containing 0.5g/litre were prepared so that 20µL contained IOµg.

Method.

10 mg. of each hydrazone were applied 11.5 cm. from the end of the paper, so that spots 1-2 mm. in diameter were obtained.

The papers were hung in a well sealed glass chromatography tank with some of the aqueous phase of the relevant solvent system in the bottom.

It was found necessary to seal the tank well because of the volatility of the solvents; this was achieved by putting a small layer of silicone stopcock grease (Edwards High Vacuum, Ltd.) between the lid and the rim of the glass tank. It was further important to line the tank walls with chromatography paper in order to achieve saturation of the strip with aqueous phase before development; failure to do so caused severe streaking of the spots. The details of the solvent run will be seen below.

(iii) Results. The addition of the large, polar dinitrophenylhydrazine molecule to an α-ketoacid would, presumably, increase both its polarity and also the degree of ionisation of the acidic carboxyl group.

As a consequence, one might expect the hydrazones to have low solubilities in the hydrocarbons which possess a low polarity. Thus, for example, n-hexane, a major constituent of ligroin has a dielectric constant (E) of 1.89 at 20°C and E for toluene is 2.438, whilst

E for amyl alcohol = 16.0
E for n butanol = 17.8
E for ethanol = 25.7

(Figures from Handbook of Chemistry and Physics, 43rd edition. Chemical Rubber Co., Cleveland, Ohio, 1961).

In view of the above facts, it was not surprising to find that the hydrazones travelled very slowly during development of the chromatograms, whilst the solvents possessing low viscosity, ran very rapidly.

To move the hydrazones sufficiently to resolve a mixture, it was necessary to allow the solvent to overrun the paper and to drip off its end, which had been serrated to promote even flow. Because of this overrunning, no Rf values are quoted here.

The three solvent systems successfully separated α-ketoglutarate and pyruvate from each other and from the branch chain ketoacids. Pyruvate was found to produce two spots, and not one, contrary to the reports of Bush and Hockaday.

Irrespective of the solvent used, development was continued until the fastest moving hydrazones, those of the branch chain ketoacids, reached the lower end of the paper. The time required for full development varied with the solvent systems, presumably due to differences in viscosity.

Thus system LT11/A85 required a run of approximately 4 hours, whilst that for system D/A90 was 12 hours.

Considerable inconvenience was occasioned by the finding that the speed of running was extremely sensitive to temperature change, and a rise in the atmospheric temperature of 2°C caused the speed to double in some experiments. Consequently, close attention during development was necessary, in order to avoid loss of the fastest spots.

Whilst no difficulty was experienced in separating α-ketoglutarate and pyruvate hydrazones, results with the branch chain ketoacids varied somewhat.

1) Irrespective of solvent system or paper used, α-ketoisocaproate and α-keto-β-methylvalerate ran with the same velocity.

2) In system T/A75 all three branch chain compounds ran with the same velocity.

3) In systems D/A90 and LT11/A85, however, the ketoisovalerate travelled slightly more slowly than the other two compounds; experiments with systems of varying composition showed that one composed of ligroin: acetic acid: water = 20:17:3 (by volume) produce slightly better separation of keto-isovalerate. Nevertheless, this could only be achieved when small loadings of the branch chain ketoacids had been applied to the paper. Thus when 10 μg of KIV and of KMV hydrazones were applied in a mixture at the origin (equivalent to approximately 5 μg of the free acid), true separation could not be obtained, a composite streak with a waist between the two components being formed.

Since analysis of blood extracts would normally contain much greater quantities of these compounds than those alone, it is evident that separation could not be obtained.

In addition to the above observations, other objections were found to the use of this method.

1) Ligroin is of variable composition,and it might be expected that chromatographic behaviour would vary from batch to batch; the experiments described above were all carried out with one batch.

2) These solvent mixtures, especially D/A90, are particularly unpleasant .

3) These acid solvent systems decompose the hydrazones, leaving a dense yellow deposit of unknown composition, at the origin.

Bush and Hockaday (1960) noted this phenomenon, and claimed that the amount lost was proportional to the load of each hydrazone, although they

appear to have studied merely α-ketoglutarate and pyruvate.

They claim (1961) to reduce this loss by keeping the paper loading to a minimum; to do so with the ketoacids studied in this work would be impossible, because of the widely differing concentrations of the compounds in blood and to put a suitable quantity of branch chain ketoacid hydrazones from blood on the paper would result in the amount of ketoglutarate derivative being immeasurably small.

The slightly differing Rf values of KIV and KMV + KIC could be exploited to achieve separation of the KIV by using longer runs at the same time incurring the serious disadvantages of increasing hydrazone decomposition and of causing the spots to diffuse greatly.

Because of the objections outlined above, it was decided to abandon this method.

c) <u>The use of Ion exchange Papers.</u>

The phenol-formaldehyde and polystyrene based ion exchange resins have been in use for many years (Adams and Holmes, 1935; D'Alelio, 1945) but they were not used for paper chromatography except in a few relatively unsuccessful experiments when paper had been dipped in a suspension of the resin particles and allowed to dry (Lederer, 1955; Lederer and Kertes, 1956).

However, cellulose is itself a cation exchanger of very low capacity since it contains a few free carboxyl groups. Thus Harrison (1912) found that HCl appeared in the eluate after passing NaCl through purified cotton.

Successful attempts have been made to use cellulose as a matrix to carry ion exchange active groups.

The cellulose molecule consists of D-gluco-pyranose units joined by

1,4 glycosidic bonds, and it is to the five -OH groups of carbon atoms, 1,2,3 and 6 of the glucopyranose residue that the substituents have been attached, although substitution is thought not to have occurred homogeneously either throughout the cellulose molecule or within the glucopyranose residues (Balston and Balston, 1961).

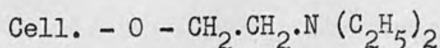
(i) Ion exchange papers available. Both cation and anion exchangers are now available, and the cation exchangers have been successful in the separation of proteins (Sober and Petersen, 1954). However, the present problem is that of separating large complex anions, and it is the anion exchangers which are of primary interest.

Anion Exchangers.

1) Aminoethyl-cellulose ("AE") This compound may be represented as cell.-O-CH_2.CH_2.NH_2 and as a primary amine it possesses very weakly basic properties. At pH values higher than 9.0 it does not exhibit any anion exchange properties.

The stability of the material to alkalis or acids which may cause scission is very high.

2) Diethyl-aminoethyl-cellulose ("DEAE") This substance is a substituent of AE cellulose:



$$\text{Cell.} - O - CH_2.CH_2.N\,(C_2H_5)_2$$

Being a tertiary amine it is more strongly basic than AE, but its other properties resemble those of AE.

3) "Ecteola" cellulose. This is a complex exchanger prepared by the reaction between cellulose, epichlorhydrin and triethanolamine. It contains tertiary amino groups, but is more weakly basic than DEAE.

(ii) Choice of Exchanger and of its form. Although both carboxymethyl cellulose (a cation exchanger) and DEAE cellulose had been available

for several years in the form of powder and floc, and had been used successfully in the separation of proteins by column chromatography, it was not until 1959 that the material made into sheet form was used to separate aminoacids by Knight (1959a, 1959b) and this form became commercially available in the latter part of that year.

At this stage of the present work only AE and DEAE were commercially available, ECTEOLA cellulose being scheduled to be marketed later. The use of these papers for the separation of the hydrazones was explored. Unfortunately little information was available at this time about the properties of the material, the valuable review of Jakubovic and Knight only becoming available in late 1960.

On general principals, however, it was assumed that the maximum capacity of the exchangers would be in the acidic range of pH, a fact subsequently confirmed by Jakubovic and Knight (1960).

In addition, these papers have low exchange capacities, and the recommendation of Knight (1960) was followed, buffers of low molarity being used.

(ii) Experimental Details.

Choice and state of papers. Whatman AE "30" of nominal exchange capacity 0.6 meq/g. and DEAE "20", nominal capacity 0.4 meq/g. were used.

The papers were used in 2 states:

1) Paper received from the manufacturers, without modification, was spotted with the synthetic hydrazones and the chromatogram developed as described below.

2) Cycled paper: Three inch wide strips of paper were washed with 0.1 N.NaOH for 24 hours, after which it was washed with distilled water until the effluent was neutral to B.D.H. narrow range indicator paper.

This usually took 12 hours. Cycling was conveniently carried out by treating the strips as descending chromatograms, the NaOH being added to the trough of the chromatographic tank. Washing was undertaken by sucking off the NaOH and adding water to the trough, after rinsing four times.

The strips were dried at room temperature for 48 hours, after which they were used in the same way as the untreated strips.

Buffers: These were all 0.1 M., except where otherwise stated. $KH_2.PO_4/Na_2H.PO_4$ buffer was used for pH values above 6, Na acetate/acetic acid at lower values.

Knight (personal communication) recommended that both the AE and DEAE papers be used at pH values less than 8. Accordingly,two phosphate buffers (pH 7.12 and 6.0) and two acetate buffers (pH 4.7 and 3.7) were prepared; the pH values were measured using the conventional type of glass electrode pH meter.

Development of the Chromatogram.

10 μg of the hydrazones in fresh aqueous solution were applied to a line 10 cm. from the end of a 7.5 cm. wide strip of ion exchange paper using conventional methods (described above).

The paper was then hung in a sealed glass chromatography tank in the usual arrangement for descending chromatography. It was left overnight to saturate with water and the following morning one of the buffers described was introduced into the trough.

First series.

The papers as they were received from the manufacturer were spotted with the hydrazones and chromatograms on DEAE and AE paper developed in

parallel by the addition of buffer to the trough. In both cases the solvent was allowed to overrun the paper, since it ran very rapidly, whilst the solute spots ran slowly . In all, 4 experiments were performed using the buffers described above.

In order to distribute the various spots down the length of the paper, it was found necessary to allow the solvent to run for 18-24 hours, by which time the most rapid spots had travelled some 30 cm.

The speed of travel resembled, in most respects, those in conventional systems i.e.

increasing speed ↓	α-ketoglutarate hydrazone Pyruvate hydrazone. Branch chain ketoacid hydrazones.

However, several observations are of considerable importance.

1) Pyruvic hydrazone, well separated from that of α-ketoglutarate, showed marked separation into two spots, the faster of which travelled close to the branch chain ketoacid hydrazones.

2) The KIV spot travelled slightly faster than KIC and KMV, both of which possessed the same velocity.

3) A disappointing finding was the severity of streaking which occurred in all spots. The length:breadth ratio of the spots of the developed chromatogram was approximately 3:1 and was such that KIV hydrazone remained unseparated from the other two branch chain ketoacid hydrazones when the chromatogram was fully developed, and the faster pyruvate spot became incorporated in the spot of the branch chain ketoacids.

The above observations applied to all four buffers, and differences in pH appeared to have no effect on chromatographic behaviour of untreated exchange paper; accordingly, it was decided to use the paper in its $-\bar{OH}$ form.

Second series.

The same experiment as in series 1, but using paper in its OH^- form, was carried out.

The results were similar to those of the first series except that streaking was much more severe, with a length:breadth ratio of approximately 7:1, so that it was not possible to achieve separation.

Before abandoning the use of ion exchange paper for this project, a few additional experiments were undertaken:

1) The molarity of the buffers was increased, first to 0.1, and then to 1.0, with no effect on resolution.

2) Although practical difficulties made it impossible to attain true gradient elution, a chromatogram was allowed to run for 10-12 hours, after which the trough was emptied and either

a) a more molar buffer solution of the same pH was introduced or

b) a buffer of the same molarity but lower pH was introduced, or

c) 1.0 M. NaCl solution was introduced.

Steps (a) and (b) had no effect on resolution, and (c) merely resulted in very wide diffusion of the spots.

Clearly the full potential of this new material had not been explored, but it was not felt justifiable to continue work on this aspect of the problem. Accordingly this type of chromatography was abandoned, but because of its probable future importance, some aspects are discussed here.

It would appear that the most likely explanation for the streaking noted on the chromatogram is that adsorptional forces have a very great effect on the chromatographic behaviour of the compounds, in addition to

any possible ion exchange effect.

Thus Davies (1949) and Davies and Thomas (1951) found that molecular adsorption effects were great in the case of the chromatography of amino-acids, organic acids or bases on ion exchange columns, and that the effect increased with rising molecular weight.

This observed cause may explain the higher speed of travel of the KIV hydrazone, which has a smaller M.W. than KMV and KIC.

Moore and Stein (1952) have noted that the rate of movement on a column of polystyrene exchanger is dependent upon affinity of the resin for both the ionic and non-ionic portions of a molecule, the latter force being determined by adsorptional forces and by the degree of hydration of both solute and column.

Jakubovic and Knight (1960) have found that incorporating water miscible organic solvents into the aqueous buffer solutions used for development has produced more compact and definite spots of amino acids.

These authors think that the introduction of the solvent adds partitional and other effects to the ion exchange effects, and they have suggested that the factors affecting separation on polystyrene found by Moore and Stein (1952) also affect ion exchange celluloses.

Jakubovic and Knight (1960) report having explored the use of buffers with organic solvents only tentatively, and, perhaps, a re-examination of the problem might yet produce a method for the separation of one, at least, of the branch chain ketoacids.

The reason for increased streaking after cycling the paper is obscure.

Perhaps the most likely explanation is that adsorptional forces are increased, and that converting to the OH^- form changes the degree of

hydration of the exchanger, which is an important factor controlling affinity of solute for exchanger.

The method finally chosen is described below.

d) The method chosen.

Considerable time was expended in the unsuccessful exploration of the methods described above,and it was decided that it was necessary to use an already established method,despite its inadequacy; the most comprehensive was that of de Schepper,Parmentier and Van der Haeghe(1958) and this method was explored with a view to its use.The wedge shaped strips used by these authors were found to be inconvenient to use,and since the method offered no better separation than conventionally shaped strips,it was decided to use parallel sided strips 10cm. wide and 40cm. long,downward development being undertaken. In all other respects,the method used here is exactly as described by the original authors.

The various steps of the method as it was finally used,and the details of each step,with particular attention to those precautions necessary for the achievement of reproducibility,are described below.

I. Collection of samples.

a) Blood

Blood was drawn from a vein into a sterile,dry syringe at room temperature,and with no fist clenching. On occasions when a tourniquet had to be used,sufficient blood was withdrawn within 90sec. of application.

After withdrawal,the blood was immediately ejected into precipitant as a thin stream ,and shaken briskly.

Tungstic acid was chosen as precipitate for several reasons:-
1. Trichloracetic acid filtrates from deproteinised blood,if kept overnight in a refrigerator,give a falsely high level of keto-compounds,apparently derived from the T.C.A. itself(Friedemann and Haugen,1943;Goodwin and Williams,1952)

Further,the precipitant destroys pyruvic acid if left overnight in mixtures.(Neish,1957)
2. Metaphosphoric acid causes emulsion formation,when extracts are shaken with organic solvents(El Hawary&Thompson,1953)
3. Tungstic acid has been widely described as a satisfactory precipitant (Seligson&Shapiro,1952;Klein,1941;de Schepper,Parmentier and Van der Haeghe, 1958;Taylor & Smith,1955; Cavallini & Frontali,1954),although the last authors noted occasionally that emulsion formation when the "protein free" supernatant was shaken with ether for extraction purposes. They thought that"it was probably due to incomplete deproteinisation", an impression

confirmed by the present author,for emulsion formation only occurred when the amount of blood was greater than that which the tungstate could deproteinise; under these conditions,a white,filmy deposit (?protein) formed at the ether/water interface. This problem was easily overcome by ensuring that a slight excess of precipitant was always present in the tubes into which the blood was ejected.

Preparation of the Precipant.

I. 2/3N. sulphuric acid was prepared and stored separately.

2. I0% (w/v) sodium tungstate (Analar) was prepared and stored separately.

3. Distilled water.

De Schepper,et al recommended a composition of:

blood: tungstate: sulphuric acid: water I:I:I:3 (by volume),

but it was necessary to compensate for an unduly alkaline batch of sodium tungstate by using more acid,and the optimum composition was found to be:

blood: tungstate: sulphuric acid: water I:I:I.2:3 (by volume).

The volume of blood usually deproteinised was 5ml.,but sufficient precipitant was put into the tubes to deproteinise 6ml. of blood,thus ensuring completion of this proceedure.

Initially the blood was lysed by the correct quantity of water and sulphuric acid, after which the sodium tungstate solution was added,but this method caused inaccuracy in the estimation of the quantity of blood used. At this stage of the study, 6" by I" glass stoppered test tubes were weighed,and I8ml. of distilled water with 7.2ml. Of the sulphuric acid were added to the tubes,after which they were weighed again.After the addition and lysis of the blood,6.0ml. of tungstate were added,the whole shaken,and reweighed. Thus any error in the measurement of the tungstate was immediately reflected in the weight of blood taken,since the latter was obtained by difference.Errors were found to be frequent in the early phases,and the method was therefore slightly modified. In order to avoid the labour of multiple pipetting when preparing the tubes, 0.2 N. sulphuric acid was prepared,and 25ml. of this solution added to the tubes , instead of (7.2ml. of the 2/3N. sulphuric plus I8ml. distilled water).

The weighed tubes were filled with both the ready-diluted sulphuric acid and the sodium tungstate solution,after which the tube was weighed again.

Thus, in summary, preparation of the tubes entailed four steps:

I. Weigh tube.

2. Add 25ml. of the 0.2N. sulphuric acid.

3. Add 6.0ml. of I0% sodium tungstate.

4. Weigh again.

The tubes prepared in this way were then taken to the bed side, where the blood was added in a thin stream, and the whole shaken vigorously. Provided that the investigator did not omit these last two precautions, this proceedure did not appear to prevent successful precipitation of the blood proteins, and it avoided the necessity of trying to add exactly 6.0ml. of tungstate under circumstances which made the possibility of an error in pipetting great. The mixed precipitant remained stable for 3-4 hours, after which a white precipitate of tungstic oxide started to form.

After collection in the above manner, the precipitated protein mixture was allowed to stand for I0min. at room temperature.

b) Urine

Urine was collected into a clean bottle containing 0.5ml. of 20N. sulphuric acid for each I00ml. of urine.

The whole sample was kept at 4°C. in the refrigerator, until used for analysis, the usual time interval being 4-5 hours.

The urine was deproteinised according to the instructions of McArdle(I957). Where the S.G. was I.02 or more, 6ml. of urine were added to 26ml. of 5% metaphosphoric acid, but where the S.G. was below I.02, increasing quantities of urine were added to decreasing quantities of more concentrated metaphosphoric acid, the amount of urine depending upon its S.G., in such a way that the final concentration of MPA was not less than 4%.

In practice, this precautions was unnecessary, since the samples studied were morning collections, in which the S.G. was never less than I.020.

2. Formation of the Hydrazones.

The deproteinised blood or urine samples were centrifuged at I,000 G for I0min., and the supernatant filtered to remove particles of precipitated protein which had been held up by surface tension.

A known aliquot of the filtrate, usually 20ml., was pipetted into 6" by I" glass stoppered pyrex tubes, and 2.0ml. of 0.I% dinitrophenylhydrazine in 2N. HCl added.

The mixture was allowed to stand for 30 minutes or longer, at room temperature, after which extraction was commenced.

(A dilute solution of DNPH in HCl was chosen because it was possible to make it up into solution at room temperature, avoiding the production of the benzotriazole artefact caused by heating. The quantity of concentrated HCl(Analar) required to give a final concentration of 2N. was taken, and 2,4 dinitrophenylhydrazine added, ground with the HCl and left overnight. Water was added to the required concentration the next day. The solution was stored in a polythene bottle.)

<u>3. Extraction of Hydrazones.</u>

The superiority of diethyl ether over other solvents has already been mentioned in Part I, and "Peroxide Free" ether (May & Baker) was used. Unlike ethyl acetate, ether causes no emulsion formation with blood extracts, but it does so with those of urine, when it was easily broken with centrifugation.

Unfortunately, ether is so volatile that it proved impossible to achieve quantitative transfer of the supernatant ether to the flask in which it was to be evaporated, by the use of an ordinary Pasteur Pipette. It was also difficult to avoid carrying over water with the ether supernatant. These problems were solved by the construction of a small scale suction transfer device (suggested by Dr. S. C. Frazer).

Yellow polythene stoppers (B I9 size, from W.J.Elliot & Co., Pontypridd) fitted the IOOml. flasks used for evaporating the etherial solutions and the red disc in the top was easily removed to reveal the hollow structure common to the larger sizes of these stoppers. The metal components were conveniently obtained from clean hypodermic and lumbar puncture needles:

I. A fine hypodermic needle was introduced through the flat lower surface of the stopper and thereafter the sharp bevel was filed so that its point and sharp edge were removed, thus avoiding the splitting of the No. I or No. 2 bore "Sterivac" polythene intravenous cannula (Allen & Hanbury, Ltd.) which was pushed over the bevel until a tight fit was achieved. After attaching the cannula, the final position of the inlet needle was adjusted , and the butt end of the needle carefully removed in such a way as to avoid

distorting the lumen; this precaution, combined with the careful filing of the end left, resulted in an even, smooth flow of ether into the flask, avoiding turbulance and splashingg.

2. A wide bore needle was inserted through the flat stopper end from above, so that its point was 3-4mm. below the lower surface.

3. The length of the polythene cannula was adjusted to an acceptable value, and a lumbar puncture needle, treated in exactly the same way as the fine inlet needle, was inserted into its end.

This relatively simple piece of apparatus allowed accurate, quantitative transfer of supernatant ether with no losses andaa minimum of unwanted water. The total capacity of lumbar puncture needle, cannula and inlet needle was not greater than 0.2ml., and it was rinsed after each extractions, by sucking 2.0ml. of ether into the flask. Figure I3 shows a sketch of the stopper, and it may be seen in use in figure I4.

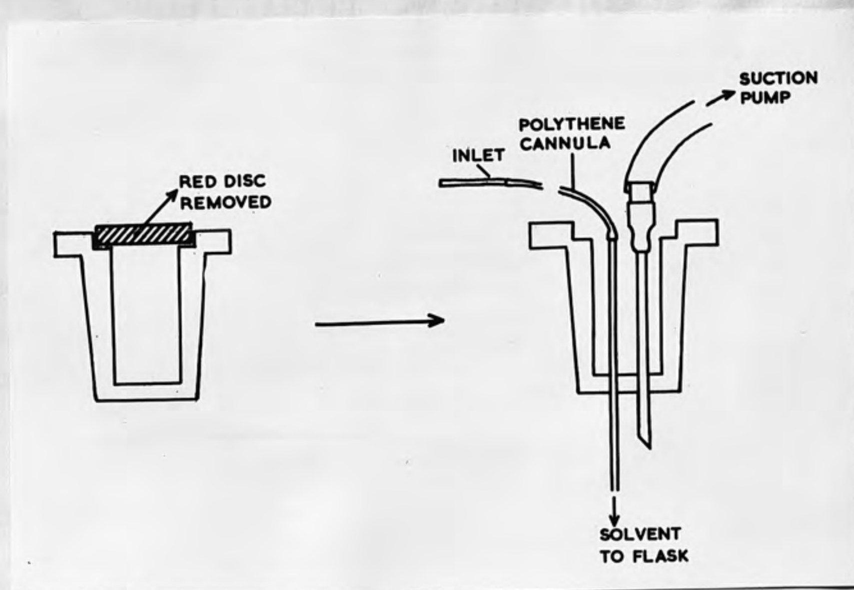


Fig. I3. The Suction Transfer Stopper.

Fig. I4. The Suction transfer Apparatus in use.

Repeated extraction of the 22ml. of reaction mixture was undertaken by shaking with ether in the glass stoppered tubes. The tubes were left for 3-4 minutes, to allow the layers to separate, and by extracting four tubes serially, a "flow" system was established, and no time wasted between each extraction. Five IOml. aliquots of ether were found to be most satisfactory, the final aliquot being colourless after shaking with the aqueous layer.

After the final extraction, the polythene cannula was rinsed with 5.0ml. ether, and the walls of the flask were washed with a further 5ml. in order to wash any solute deposited high on the flask wall (due to splashing) was washed to the bottom.

Using this method of transfer, losses appeared to be constant and small.

4. Evaporation of the Etherial Solution.

A ground glass connector piece was inserted into the flask and the whole was transferred to the suction apparatus shown in Fig I5. Here the flask contents were evaporated under reduced pressure in an atmosphere of nitrogen, at 25°C.

By adjusting the nitrogen "leak" and the suction, rapid evaporation without boiling and splashing could be obtained, and the ether was removed in 20 minutes, the residual water requiring a further 45min.

Since no other published method mentions the use of nitrogen during evaporation, it is worth noting that it was impossible to achieve reproducibility of results when the ether solution was evaporated with an air leak, but the introduction of a nitrogen atmosphere resulted in an immediate and dramatic improvement, to give the degree of precision noted below.

Presumably the hydrazones are decomposed by oxidation, under the circumstances described above, although the nature of the process has not been studied further. Smith(I960) notes that "prolonged evaporation, even in nitrogen, has been reported to decompose certain hydrazones, particularly of pyruvic acid", although he gives no reference to the relevant literature.

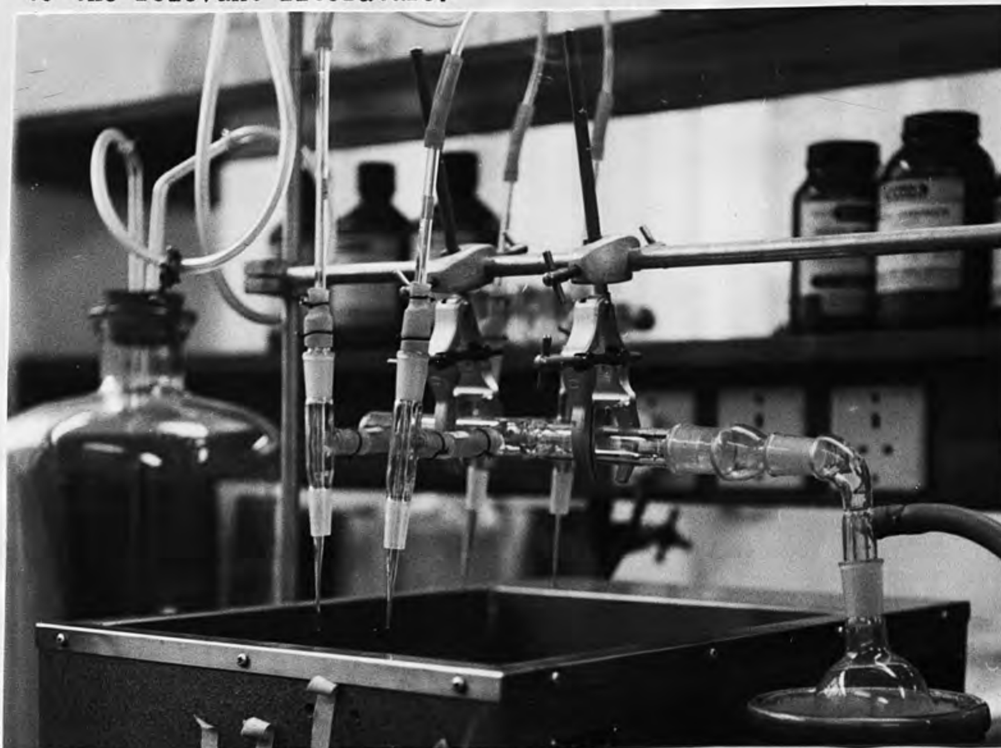


Fig. I5 The Evaporation Apparatus.

5. Preparation of the residue for Chromatography.

I.Oml. of I.ON. ammonium hydroxide was added to the flask containing the dry residue,and the walls wetted in order to take up the acidic hydrazones. It is of great importance that all areas be wetted,the latter being achieved by swirling the flask. Unless the flask walls are grease free,it is not possible to achieve the efficiency of wetting required, and it was found necessary to boil the flasks in concentrated "Diversey Pyroneg" (Deosan,Ltd.) for 20 minutes after each use. Swirling must be continued for AT LEAST five minutes,after which small particles,probably of neutral hydrazones or of free DNPH, remain undissolved.

I.Oml. of Analar chloroform was added to the flask,and the whole carefully swirled until the remaining particles dissolve.The mixture of phases was then poured into a I5ml. conical tube,and centrifuged for 5-IOmin. at full speed in a small M.S.E. centrifuge.

6. Preparation of Chromatography Paper.

50cm. by IOcm. paralleledged strips of chromatography paper were used,and the characteristics of Whatman Nos. I,4 and3MM papers were studied,in addition to the effect of buffering the papers at different pH values.

The strips were buffered by dipping them in a trough containing O.IM sodium barbitone/HCl buffer at pH 7.0,8.0,8.6 or 9.0,drying at room temperature for 24 hours before use.

Although buffered paper produced more compact spots than the parent papers,they had little effect on separation of the individual keto-acid hydrazones. Since pH 8.6 appeared to produce the most compact spots,it was decided to use this buffer.

Of the papers, No.I proved to be most satisfactory; the fast No.4 paper produced rather more diffuse spots than did 3MM and No.I,and a study of the recovery of known quantities of hydrazones after chromatography showed the recovery from No.I to be 3% higher than that from 3MM.

To obtain reproducible results, it is particularly important that the area of corresponding hydrazone spots in different developed chromatograms be equal. Thus in one of the early experiments,equal quantities of pyruvic hydrazone were applied to separate papers,but after development one area was some 40% larger than the other,and produced a I5% lower recovery.

It was not difficult to achieve equal areas in practice,by maintaining the conditions under which the ammoniacal supernatant was

applied to the paper constant,and by applying it to a line at the origin of constant length (3.7cm.)

The relatively large volumes of supernatant were applied to the origin bymmeans of a chromatography pipette made by drawing out the tip of a graduated I.0ml. pipette to a fine point,in such a way that the proximal bore was undistorted ,and it could still be used to measure the volume of supernatant applied.The tip was bent,and a fine polythene tube applied,itself being drawn out to a fine point which was lightly smeared with silicone stopcock grease to prevent solution running along it in a retrograde direction. A sketch diagram of the pipette tip is seen in Figure I6.

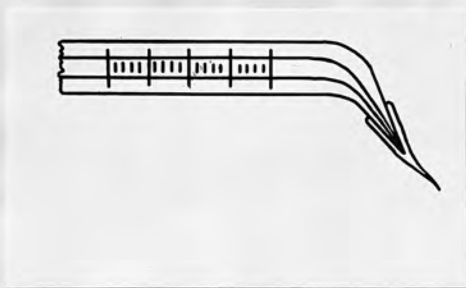


Figure I6. The chromatography pipette.

Using this pipette,the desired volume of ammoniacal supernatant was applied to the paper,using a stream of compressed,filtered air at room temperature. 0.35-0.50ml of blood extract,or 0.I-0.2ml of urine extract were the usual volumes,and 0.35ml. could be applied in 30 minutes.

The strip was suspended in an all glass chromatography tank and left overnight for it to equilibrate with the vapour of the aqueous phase,after which the organic phase was introduced,and descending development carried out for I2 hours. The tanks,and later the strips ,when drying,were kept in the dark,since pyruvic hydrazone is reported to be sensitive to light(Markees,I955).

7. The solvent system used.

None of the convential systems has advantages over any other, so that the one of de Schepper,Parmentier and Van der Haeghe(I958) was used;

n-butanol: ethanol:water 4:I;5 (by volume).

The mixture was kept in a separating funnel for 3-4 days before use. A small proportion of the lower aqueous phase was added to the

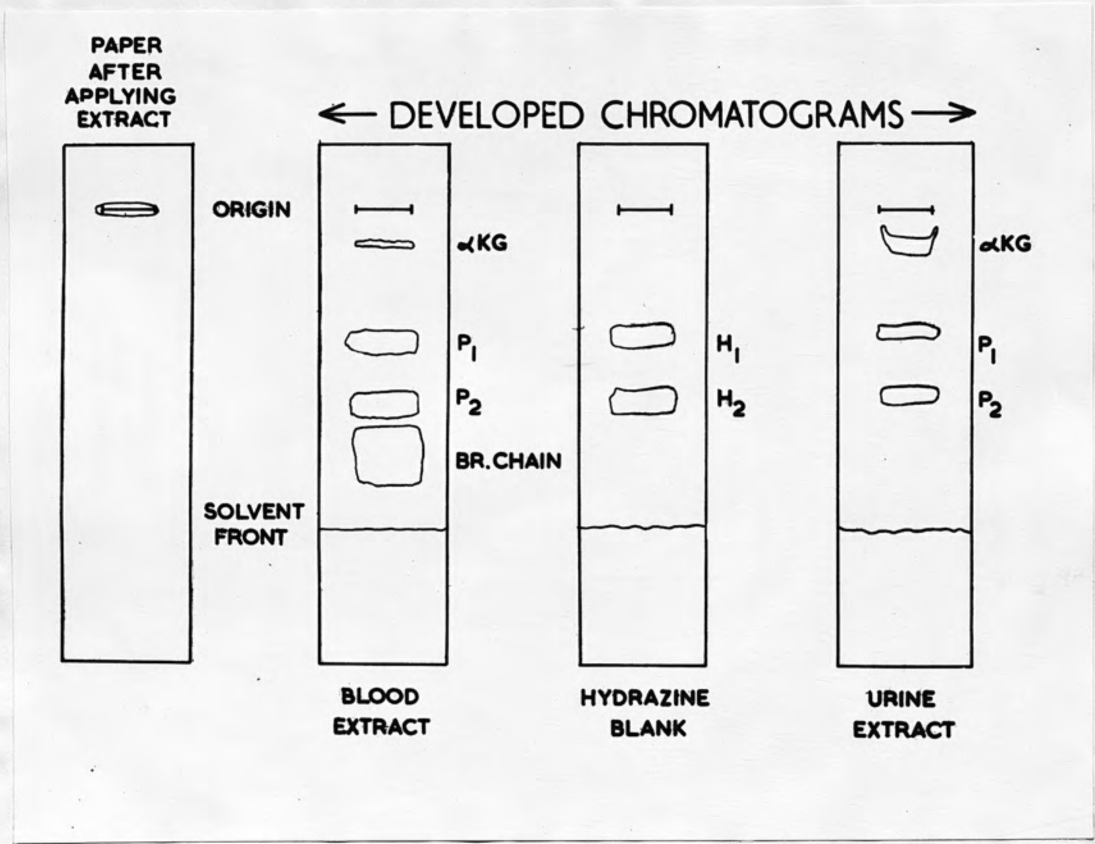


Figure I7. Diagram of the chromatograms of keto-acid hydrazones.

bottom of the chromatography tank, the remainder being kept in contact with the butanol phase; failure to take this precaution resulted in streaking of the spots during development.

The Rf values observed in many experiments are presented below,

Parent Keto- acid.	Rf value of hydrazone	
	From blood extract	From pure keto-acid solution
α-ketoglutaric	0.I4-0.I9	0.I5-0.20
slow pyruvic	0.46-0.56	0.44-0.54
fast pyruvic	0.59-0.69	0.57-0.67
KIV	---------	0.86-0.9I
KIC	---------	0.84-0.94 (elongated spot)
KMV	---------	0.84-0.9I
Branch chain spot	0.78-0.89	---------
Aceto-acetate	---------	0.84
Free DNPH, slow	---------	0.45-0.55
" " , fast	---------	0.59-0.68

It will be seen that considerable variations occur in the Rf value of any one keto-acid, attributed by many to slight variations in solvent composition, temperature, etc. (Smith, I960). Nevertheless, in any one chromatogram, the α-ketoglutarate, the two pyruvate, and the "branch chain" spots are well separated, and a diagram of the chromatograms may be seen in Figure I7.

Confirmation of the identity of each spot was made by carrying out chromatography on the ammoniacal extract of two aliquots of the same serum, to one of which was added a pure sample of the keto-acid in question; on eluting the spots, those corresponding to the keto-acid in question were shown to have a higher concentration on the "test" chromatogram.

It will be seen that free 2,4-dinitrophenylhydrazine travels with speeds equal to those of the pyruvic hydrazones, so that it is always necessary to include a "hydrazine blank" when studying the keto-acids with this method.

Additional confirmatory evidence for the site, in the developed chromatogram, of the various hydrazones may be obtained by the use of:

The alkali dip reagent (Stewart,1953)

After dipping the developed chromatogram in a 2%(w/v) solution of sodium hydroxide in 90% ethanol,the individual keto-acid hydrazones develop a colour characteristic of some of them.

Chromatograms were prepared from the pure keto-acids hydrazones which had been prepared above,and after development the alkali dip reagent was applied.

Synthetic hydrazone spot	colour developed with the dip reagent.
α-ketoglutarate	green-yellow
slow pyruvate	brown
fast pyruvate	yellow
KIV	brown
KMV	brown
KIC	elongated spot,with a yellow front part,and a brown rear portion.
slow free DNPH	yellow
fast free DNPH	yellow

The dip reagent was afterwards applied to a chromatogram prepared from blood extract; the α-ketoglutarate spot gave the expected colour,but the spot corresponding to the slower pyruvate isomer produced a spot which was entirely brown except for a narrow upper border which was yellow,presumably due to the free DNPH travelling with the pyruvic hydrazone. The faster pyruvate spot was entirely yellow,but the "branch chain spot" presented an interesting appearance; the spot was very diffuse,spreading a considerable distance beyond the margin of ultraviolet opacity,in the direction of solvent flow,and its colour was brown,except for a narrow band running across its centre,which was yellow. Since KIC hydrazone produces an elongated spot whose extemities are different colours (and due to the formation of isomers,as discussed in Part I of this thesis) ,the most likely explanation of the appearance of the branch chain spot would seem to be that the slowest,brown band and the yellow band are due to the isomers of KIC hydrazone,whilst the fast brown band is due to both KMVand KIV hydrazone together. This suggestion is in keeping with the experience that the long streak of KIC

hydrazone travelled slightly more slowly than those of KMV and KIV in experiments in which the chromatographic behaviour of the individual compounds was examined.

8. Elution of the spots.

After development, the papers were dried overnight at room temperature in a darkened room, and the outline of the hydrazone spots marked with a pencil, using transmitted ultraviolet light ("Chromatolite", Hanovia, Ltd.).

The spots were cut from the paper in such a way that there is a border of uncoloured paper around the pencilled margin, particularly in the case of the "branch chain spot", where diffusion beyond the pencilled margin was found to be marked (see above).

Elution was carried out by rolling the strips carrying the hydrazones into loose coils, placing them in the bottom of test tubes and adding the required volume of I.ON. NaOH. By using this technique, both surfaces of the paper were wetted by the sodium hydroxide, and elution was most efficient; quantitative studies of elution of known quantities of hydrazones from chromatograms showed that a higher recovery was obtained by using this technique, than by using the usual, conventional method of cutting the spots into strips, which appeared to prevent efficient wetting of the paper, because the strips lay against each other, even when the tube was shaken. The coil was allowed to stand for 5 minutes, before it was pulped using a fine glass rod, after which the pulp was mixed carefully. The pulp was allowed to stand for a further 5 minutes.

This elution process must be carried out with great care, in order to obtain maximum recovery. During preliminary experiments, it was found that the efficiency of elution was increased when the NaOH:paper ratio was increased; however, addition of a large volume of sodium hydroxide solution produced a very faint pink colour, and it was necessary to strike a compromise; and the volumes of NaOH chosen to elute each spot are presented below:

Hydrazone	Volume of NaOH to elute(ml.)
βα-KG (from blood)	2.0
α-KG (from urine)	5.0
Slow pyruvate	5.0
Fast pyruvate	5.0
Branch chain K-As	7.0

The pulp was centrifuged at I,200G for 5 minutes,after which the tube was vigorously shaken,in order to dislodge particles of paper which were held at the water surface.Centrifugation with the same conditions as above was repeated. Without this "double centrifugation" technique,paper particles were introduced into the spectrophotometer cell and falsely high readings obtained.

9. Estimation of the Hydrazones.

After centrifugation, the supernatant solution was removed with a Pasteur pipette,and introduced into the silica micro cells (Icm. light path) of a Unicam SP 500 spectrophotometer.

The optical density of the pink colour was measured against a blank prepared by eluting a corresponding area from a chromatogram of an ammoniacal extract,in which the original test solution(blood or urine) had been replaced by distilled water. The optical densities were measured at 440mμ,except for α-ketoglutarate hydrazone,whose optical density was measured at 420mμ.The actual quantity of keto-acid present in the original test solution was calculated from formulae presented below.

The method has been presented step by step above,and particular stress has been laid on those features which were found to be of great importance in obtaining reproducability;it remains,however,to obtain some estimate of the precision.of the method:

I. Quantitative evaluation of the method.

The first step was to establish the stability of the pink colour of the hydrazones in sodium hydroxide solution; the pure hydrazones were dissolved in I.ON. NaOH solution,and the optical density measured at intervals up to 2 hours afterwards,and it will be seen from table 6A that,even at I20 minutes,the optical density is only slightly less than that at the time of dissolving,in the case of all hydrazones examined.

Table6A The stability of the colour in I.ON. NaOH.

Time after adding NaOH (min.)	Optical density of hydrazone.				
	α-KG	Pyruv.	KIC	KMV	KIV
2.0	.38I	.605	.405	.44I	.8I4
2.5	.382	.605	.406	.440	.8I7
3.0	.370	.605	.405	.442	.8I4
3.5	.378	.603	.407	.438	.8I6
4.0	.379	.604	.	.442	.8I5
4.5	.38I	.605	.400	.438	.8I5
5.0	.379	.605	.398	.438	.8I7
6.0	.383	.607	.395	.442	.8I4
7.0	.380	.606	.402	.442	.8I6
8.0	.380	.604	.405	.435	.8I6
9.0	.378	.602	.402	.442	.8I3
IO.0	.367	.599	.405	.439	.8I4
I5.0	.370	.605	.406	.445	.820
20.0	.372	.603	.404	.442	.8I6
25.0	.374	.604	.402	.439	.8I7
30.0	.368	.595	.398	.428	.799
60.0	.362	.592	.394	.422	.792
I20.0	.355	.583	.386	.4I5	.783

2.The Absorption Spectrum of the Hydrazones in I.ON. sodium hydroxide.

The pure hydrazones prepared above were dissolved in the sodium hydroxide,and the optical-densities determined at wave-lengths between 370mμ and470 mμ,using the Unicam SP500 spectrophotometer.

The results are recorded graphically in figures I8-22, where the ordinate, $\frac{E_\lambda}{E_{\lambda max}}$ represents the ratio:

$$\frac{\text{Optical density at W.L. } \lambda}{\text{Optical density at W.L. of maximum absorption.}}$$

It will be seen that the maximum absorption occurs,in all cases, except α-ketoglutarate hydrazone, at or about 440mμ,and the optical densities of all unknown solutions were subsequently measured at this wavelength;the exception,α-ketoglutarate hydrazone,showed a peak of absorption at 420mμ,and the optical densities of solutions of this hydrazone in NaOH were subsequently measured at that wave-length.

The absorption spectrum of free 2,4-dinitrophenylhydrazine over the same range of wave-lengths was prepared,and is presented graphically in figure 23. The absorption spectra of the different hydrazones eluted from chromatograms resembles those of figures I8-22 so closely,when measured against the "hydrazine blank" that they are not reproduced here. Similarly,the spectra of the two spots produced on the chromatograms of free 2,4-DNPH (i.e. the "hydrazine blanks",H_I and H_2) closely resembled the spectrum of the parent compound,and figure 23 is representative of all three.

The quantity of barbitone buffer eluted from the chromatograms along with the hydrazones is difficult to define,but it was thought wise to see whether the presence of a relatively large quantity of barbitone buffer had any effect on the pH of the eluate,and whether this change had any appreciable effect on the absorption spectrum.Figure I8 shoes the spectrum of α-ketoglutaric hydrazone dissolved in a mixture of NaOH and barbitone buffer;because the spectra were so very similar,it has been necessary to draw the curves on separate scales to prevent them overlapping. A similar lack of effect was found with KMV-hydrazone.

Figures 18-23 The absorption spectra of the keto-acid hydrazones and free DNPH.

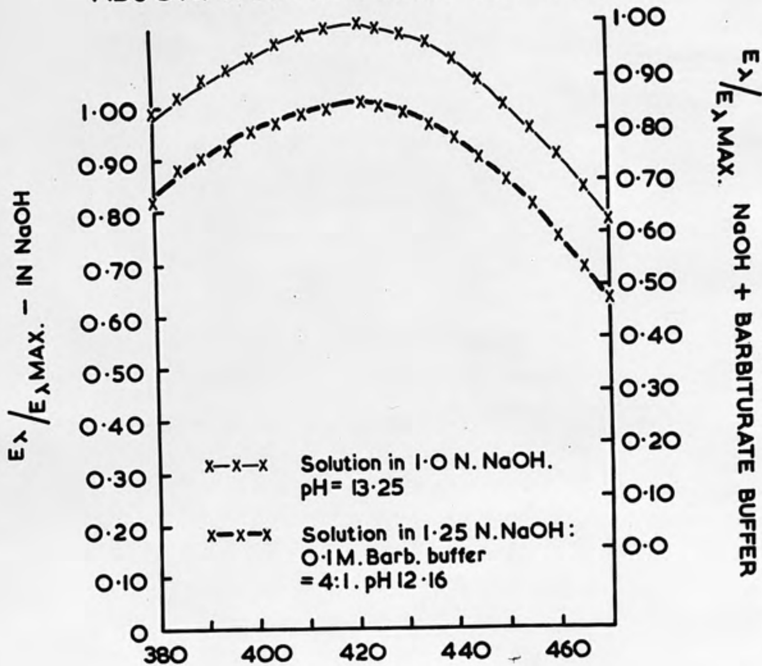


Figure I8. The absorption spectra of α-ketoglutarate hydrazone in I.ON. NaOH and in NaOH/barbitone buffer 4/I. $E_{\lambda max}$ = (NaOH) 0.525 $E_{\lambda max}$ = (buffer) 0.325.

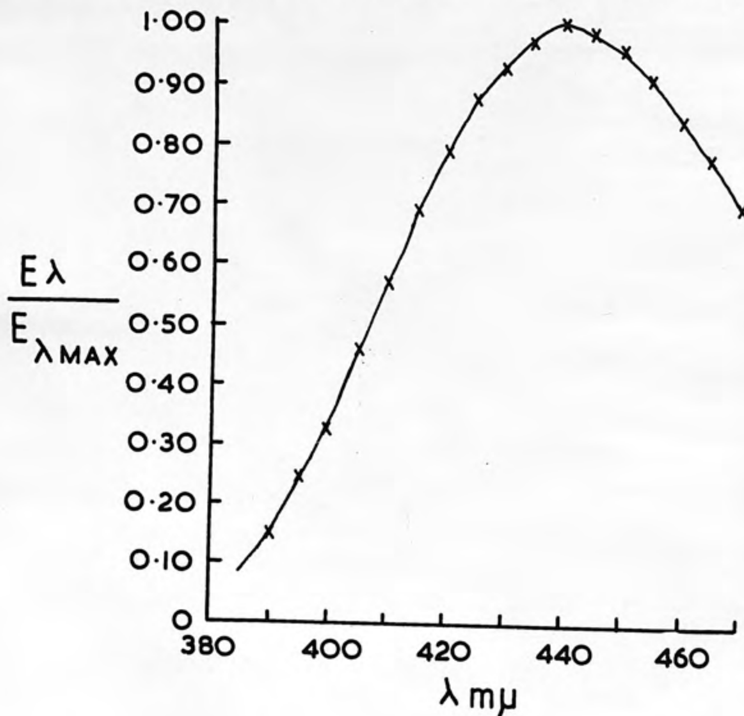


Figure I9. The absorption spectrum of pyruvic hydrazone in I.ON.NaOH. $E_{\lambda max}$ = 0.606.

Fig. 20. The absorption spectrum of KIV-hydrazone in I.ON. NaOH
$E_{\lambda max} = 0.380$

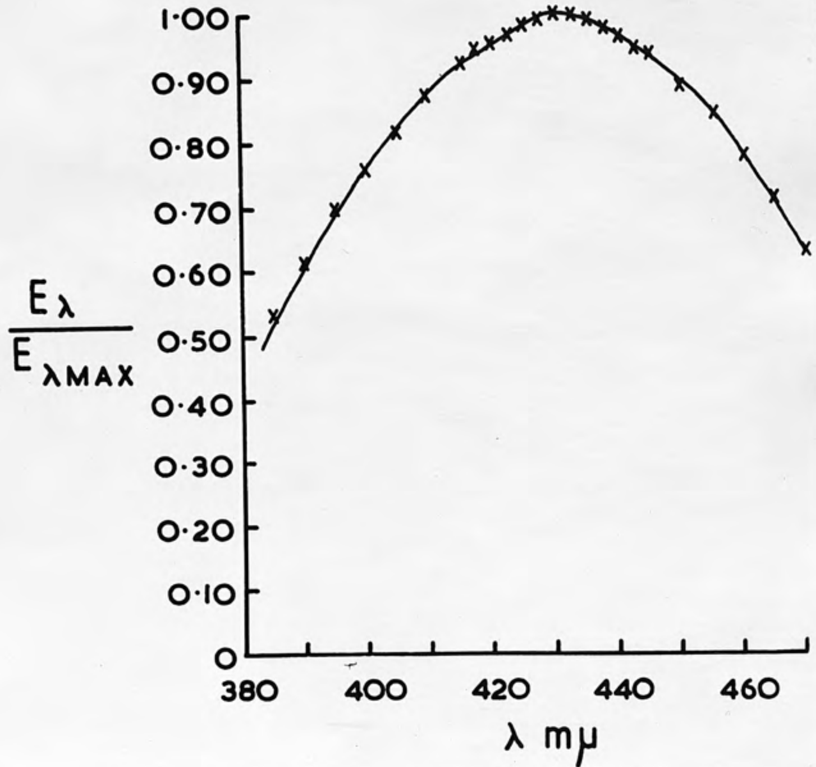


Fig. 2I. The absorption spectrum of KMV-hydrazone in I.ON. NaOH.
$E_{\lambda max} = 0.582$

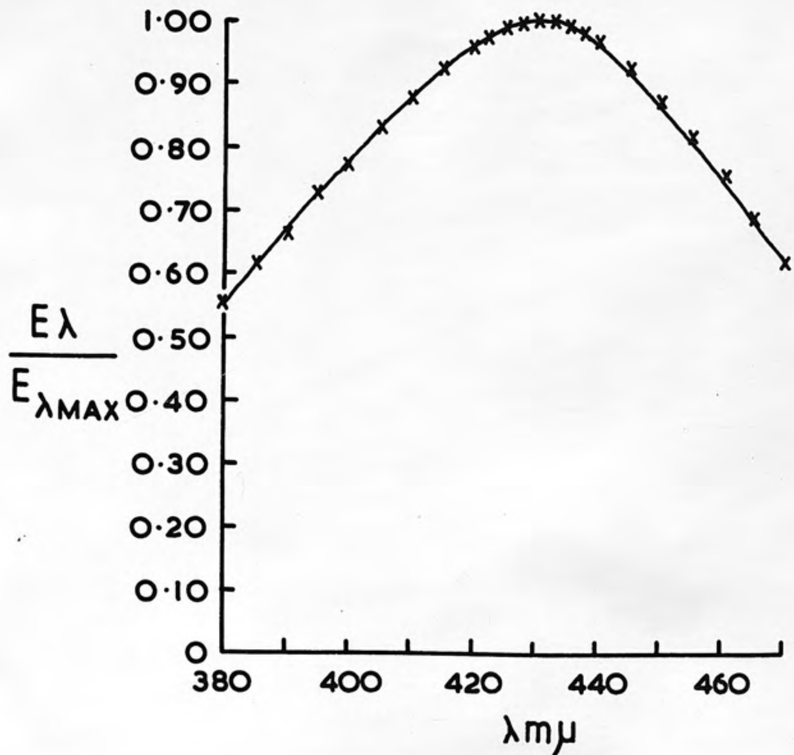


Fig. 22 Absorption spectrum of KIC-hydrazone in I.ON.NaOH.
$E_{\lambda max} = 0.347$

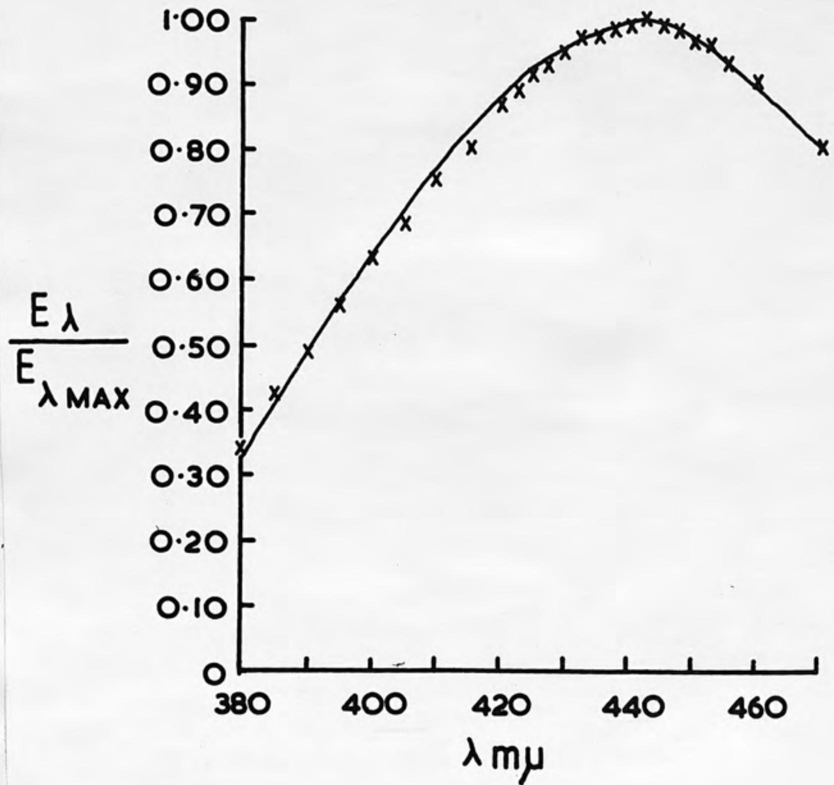
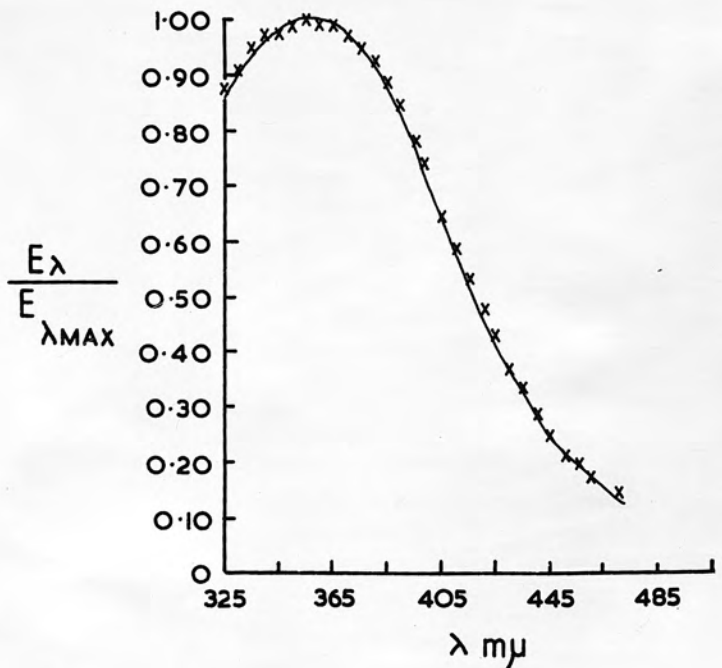


Fig. 23 Absorption spectrum of free DNPH in I.ON.NaOH



Figures 24-28 The calibration graphs for the keto-acid hydrazones.

α-ketoglutaric hydrazone.

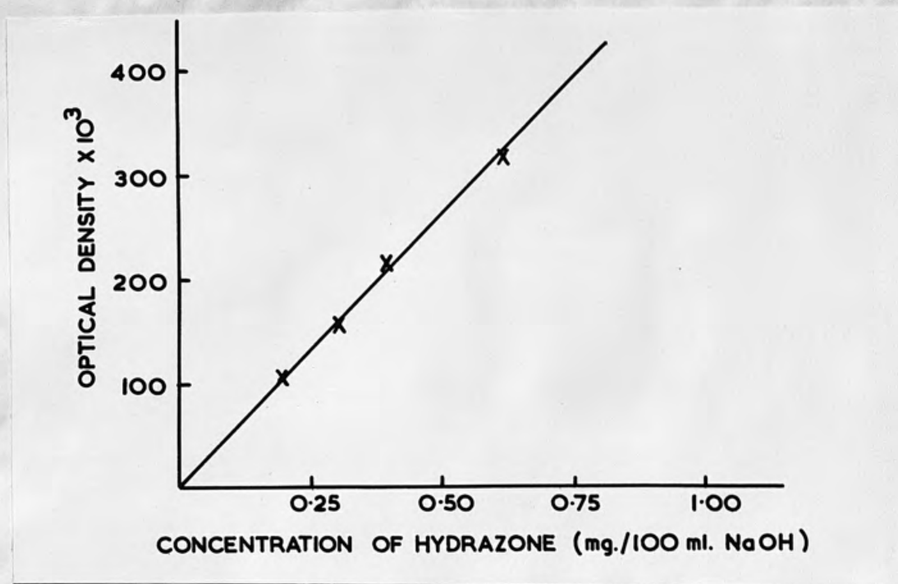


Fig.24. The calibration curve for βα-ketoglutarate(optical densities at 420mμ)

Weight of dry hydrazone66.IOmg. Dissolved in I,000ml to prepare "stock" solution.

Sample	"Stock" solution,ml.	I.ON.NaOH,ml.	Optical density*	Concentration,mg/IOOml
I	Undiluted		0.322	0.6IO
2	4.0	2.0	0.2IO	0.4I
3	3.0	3.0	0.I57	0.3I5
4	2.0	4.0	0.III	0.20

* corrected for difference between cells.

Pyruvic hydrazone.

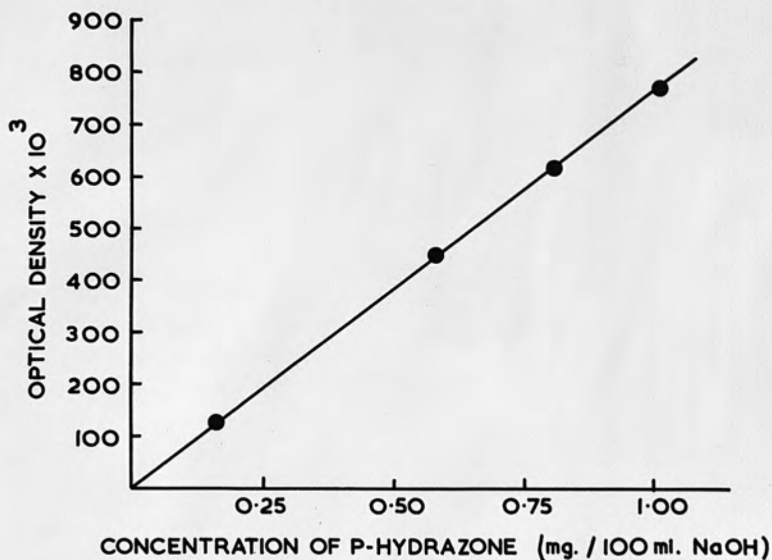


Figure25.

Weight of dry hydrazone 5.03mg, dissolved in 500ml. NaOH.

Sample	"Stock"solution,ml.	I.ON.NaOH,ml.	Optical density*	Concentration,mg/Iooml
I	Undiluted		0.770	I.0I
2	4.0	I.0	0.624	0.8I
3	2.0	2.0	0.385	0.50
4	I.0	4.0	0.I58	0.20

-* corrected for difference between cells.

Keto-isocaproic hydrazone.

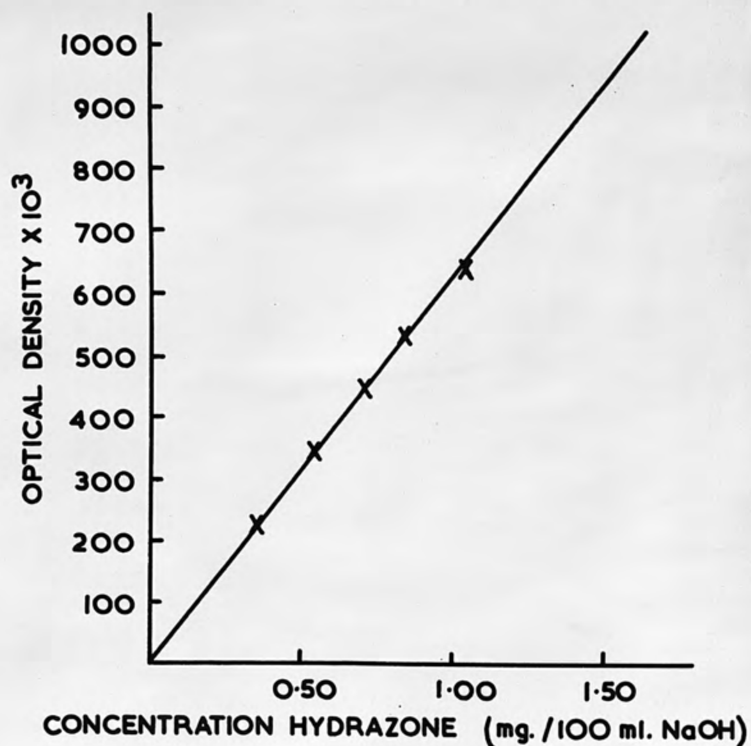


Figure26.

Weight of dry hydrazone,4.68mg;dissolved in 50ml. I.0N. NaOH to produce "stock" solution.

Sample	"Stock"soln.,ml.	I.0N.NaOH,ml.	Optical dens.*	Concentration,mg/I00ml.
I	0.50	4.0	0.638	I.04
2	0.50	5.0	0.533	0.85
3	0.50	6.0	0.455	0.72
4	0.50	8.0	0.348	0.55
5	0.50	I2.5	0.225	0.36

* corrected for difference between cells.

Ketǫ-isovaleric hydrazone.

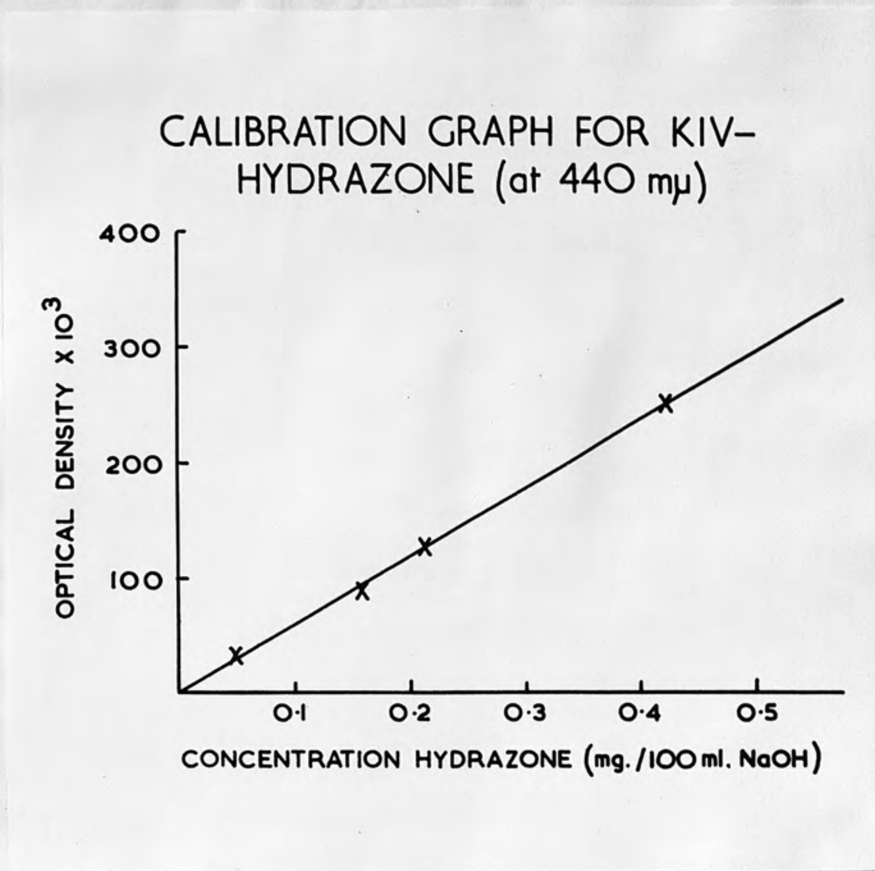


Figure27

Weight dry hydrazone 4.22mg.,dissolved in 2,000ml. of sodium hydroxide solution. This volume of sodium hydroxide was necessary because of the extreme insolubility of the hydrazone.

Sample	"Stock"soln.,ml.	I.ON.NaOH,ml.	Optical dens.*	Concentration,mg/IOOml
I	Undiluted		0.I27	0.2I
2	I5.0	5.0	0.094	0.I6
3	I0.0	I0.0	0.063	0.II
4	5.0	I5.0	0.033	0.05

*corrected for difference between cells.

α-keto-β-methyl-n-valeric hydrazone.

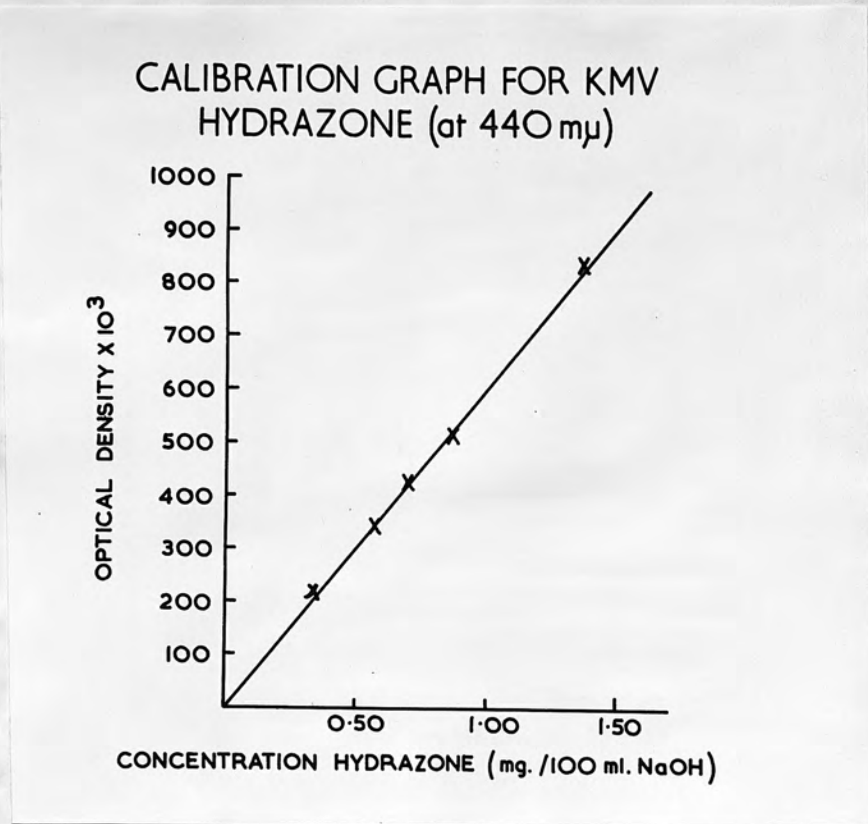


Figure28.

Weight of dry hydrazone, 3.44mg.,dissolved in 50ml. sodium hydroxide,to form the "stock"solution.

Sample	Stock soln., ml	I.ON.NaOH,ml.	Optical dens*	Concentration,mg/IOOml.
I	0.50	2.0	0.835	I.38
2	0.50	3.5	0.5I5	0.86
3	0.50	4.5	0.420	0.69
4	0.50	5.5	0.343	0.57
5	0.50	9.5	0.2I0	0.35

* corrected for differences between cells.

3.Establishment of Calibration Curves.

Pure synthetic hydrazones,prepared in section 2.I.I.,and dried to constant weight,were weighed out and dissolved in a known volume of sodium hydroxide solution,to produce a "stock" solution,which was then diluted with varying volumes of sodium hydroxide solution,to produce a range of dilutions which might be expected in extracts from biological fluids. It was necessary to take great care with the initial weighing and dissolving of the hydrazone in sodium hydroxide,since an error at this stage would be reflected in all the dilutions. After comparing the optical density of two silica micro-cells containing I.ON. NaOH,the optical densities of the varying dilutions of hydrazones was measured against I.ON. NaOH as a blank. The light path was Icm. long, and the machine an SP 500 spectrophotometer. The results for five hydrazones are presented below,in graphical and tabular form.

Since it would be inconvenient to calculate the blood concentrations on each case by tedious,prolonged computations,a formula was derived to apply to each keto-acid,and the method of calculation is presented in Appendix 2.Examination of the calibration curves (figures 24-28) shows that they obey Beer's Law over the ranges of concentration studied,and therefore it is valid to apply the formulae to calculating the concentrations in most samples of blood.

4. Reproducibility of the method.

In order that the experimental conditions should resemble normal working conditions as closely as possible,serum was used as a source of the keto-acids to be studied. Quadruplicate samples of fresh serum were subjected to the extraction and chromatography proceedure described above. Those sera in which it was thought that there may be abnormal concentrations of keto-acids(e.g. hepatic disease) were rejected. Several "lots" of 4 samples were processed for each of the keto-acids, as isolated from blood;an estimate of the standard deviation(S.D.) for each "lot" of 4 samples was obtained,after which it was necessary to see if the concentration of keto-acid in the sample had any effect on the magnitude of the S.D. This was undertaken by calculating the coefficient of regression of the S.D. upon the mean concentrations for each"lot". The results are presented here in a summarising table,but the full details,and the method of computation are presented in detail in Appendix I.

The reproducibility of the method.

Keto-acid	NO. of Lots studied	Range of Lot means (μg/100ml.)	Range of S.D.s	Range of Coefficient of variation, ± %	Significance of regression coefficient (b)
α-KG	6	*181.2-577.7	±3.0-±14.5	1.19-4.23	P* < 0.01
Pyr.	6	771.0-1881.9	±3.82-±46.74	0.88-7.60	0.1 > P* > 0.05
Br.Ch	4	1754.9-2513.8	±12.9-±17.6	2.19-3.94	0.4 > P* > 0.3

* Probability that b differs from 0 due to chance.

Unfortunately the range of keto-acid concentrations found in the samples of serum studied was higher than that of normal blood, although this fact was not discovered until near the end of the study, when sufficient data on the normal blood concentrations had been accumulated, and no one appears to have reported this higher concentration of keto-acids in serum; nevertheless, the ranges reported here, in the case of α-ketoglutaric and pyruvic acid, are of the same order as the raised concentrations observed by various authors, in some diseased states, when blood concentrations were studied. (See Part I). Only the range of "branch chain" keto-acids reported in this small study of serum appears to be very much higher than the normal blood concentrations, and whether this range of concentrations is very much different from that which would be observed in disease is difficult to confirm, because of the few reports of studies on the blood concentrations of these compounds.

The author feels, however, that the reproducibility is of an acceptable order, both in view of the dependence on chromatography, and also because of the relatively small quantity of α-ketoglutaric acid present in blood. It is interesting that only the regression coefficient for α-ketoglutaric acid is significant, suggesting that the S.D. is effected by the concentration in the serum.

5. The recovery of added keto-acids.

Known quantities of pure keto-acids were added to serum, and the whole put through the extraction and chromatography process, along with equal aliquots of the same serum to act as "serum blank".

Two samples each of the "serum blank",and serum with added keto-acid were processed at the same time.The difference between the optical density values of the final eluates from the chromatograms of these two groups represented the added keto-acid.

α-ketoglutaric acid recovery.

From the formulae calculated in Appendix 2,the relevant formula is:

$$\text{concentration of free keto-acid}(\mu g/100ml.) = \frac{O.D. \times A \times D \times 146}{m \times B \times C \times E \times 326}$$

Substituting: A = 3.0ml.; B = 0.5ml.; C = 15.0ml.

D = 31ml. (5ml. serum + 15ml. water + 6ml. H_2SO_4 + 5ml. tungstate)

E = 5ml.; m = 0.520.

$$\text{concentration of free keto-acid} = \frac{O.D. \times 3 \times 31 \times 146}{0.520 \times 0.5 \times 15.0 \times 5.0 \times 326}$$

From this expression,the estimate of the quantity of keto-acid added may be made,and the difference between this estimate and the quantity actually added represents the quantity lost during the proceedures to which the serum was subjected:

μg. pure acid added to 5ml. serum	O.D. "serum blank"	O.D. serum +α-KG	Diff. between mean O.D.s	Estimate of α-KG added, μg (from equation)	% recovery	Mean recovery of each group%
I4.09	0.228	0.327	0.I02	I0.89	77.4	
	0.222	0.327				
I4.09	0.283	0.382	0.I00	I0.68	75.8	75.7
	0.282	0.383				
I4.09	0.330	0.425	0.097	I0.40	73.9	
	0.323	0.422				
I0.I3	0.I99	0.27I	0.076	8.I2	80.2	
	0.I97	0.277				
I0.I3	0.I62	0.238	0.073	7.80	76.9	77.3
	0.I6I	0.23I				
I0.I3	0.264	0.333	0.07I	7.58	74.8	
5.I2	0.264	0.337	0.037	3.96	77.4	
	0.254	0.293				
5.I2	0.I6I	0.I99	0.036	3.84	75.2	77.2
	0.I65	0.I98				
5.I2	0.303	0.339	0.038	4.04	79.I	
	0.30I	0.339				

Recovery of pyruvate.

From Appendix 2, the relevant formula is:

concentration of pyruvate (as sodium salt)

$$= \frac{\text{O.D.} \times 10 \times 31 \times 110}{0.770 \times 0.5 \times 15.0 \times 5.0 \times 326}$$

where A = 10.0ml. B = 0.50 C = 15.0ml. D = 31ml. E = 5.0ml. and m = 0.770

The optical densities quoted are the sum of those for spots P_1 and P_2, in each case.

μg. Na. pyr. added to 5ml. serum	O.D. serum blank	O.D. serum +pyruvate	Diff. between mean O.D.	Estimate of NaPyr. added (from equation), μg.	%recovery	Mean recovery of each group, %
40.10	0.590 0.565	0.765 0.723	0.166	30.14	75.2	
40.10	0456 0.428	0.605 0.583	0.152 0.	27.6	68.9	
40.10	0.695 0.667	0.839 0.857	0.158	28.7	71.6	71.9
24.32	0.321 0.306	0.403 0.422	0.098	17.8	73.3	
24.32	0.717 0.685	0.761 0.855	0.107	19.4	79.8	
24.32	0.458	0.572	0.111	20.1	82.3	78.5
18.92	0.541 0.565	0.612 0.642	0.074	13.4	70.8	
18.92	0.503 0.475	0.633 0.599	0.072	12.7	67.2	
18.92	0.521 0.545	0.613 0.575	0.061	11.1	58.7	65.7

Recovery of the branch chain keto-acids.

Only the recoveries of KIV and KIC were studied, because of the small quantities of KMV available.

a) KIV

Substituting in the relevant formula in Appendix 2,

$$\text{concentration of free keto-acid} = \frac{0.D. \times I0 \times 3I \times II3}{0.59 \times 0.50 \times I5.0 \times 5.0 \times 293}$$

where m = 0.590, and the same volumes of serum, protein free supernatant, etc. are used, as for pyruvate.

µg. KIV added to 5ml. serum	O.D. serum blank	O.D. serum + KIV	Diff. between mean O.D.s	Estim. of KIV added (from equn.)	%recovery	Mean recovery of each group, %
60.48	0.206 0.206	0.330 0.348	0.I33	35.9	59.4	
60.48	0.32I 0.326	0.470 0.460	0.I4I	38.2	63.2	6I.3
3I.20	0.308 0.3II	0.389 0.373	0.07I	I9.2	6I.6	
3I.20	0.43I 0.420	0.484 0.502	0.067	I8.I	58.I	59.8

b) Keto-isocaproate recoveries.

In a similar way to the above,

Concentration of free keto-acid (as sodium salt)

$$= \frac{0.D. \times I0 \times 3I \times I52}{0.625 \times 0.50 \times I5.0 \times 5.0 \times 3I0}$$

µg. NaKIC added to 5ml. serum	O.D. serum blank	O.D. serum +NaKIC	Diff. between mean of O.D.s	Estimate of NaKIC added, µg. (from equation)	%recovery	Mean recovery of each group, %
74.32	0.23I 0.227	0.377 0.369	0.I44	46.7	62.8	
74.32	0.40I 0.392	0.526 0.543	0.I37	44.5	59.9	6I.4
36.3I	0.37I 0.362	0.430 0.443	0.070	22.7	62.6	
36.3I	0.332 0.34I	0.398 0.409	0.067	2I.7	59.7	6I.I

The figures presented above for reproducibility and recovery are similar to those published by other workers who used similar methods(e.g.Seligson and Shapiro,I956; McArdle,I957).

From an inspection of the results for recoveries,the corrections to be applied to the formulae to correct for losses were estimated as being:

Keto-acid(s)	overall % recovery	Correction applied.
Ketoglutaric	75	$\frac{100}{75}$
Pyruvic	70	$\frac{100}{70}$
Branch chain	60	$\frac{100}{60}$

The branch chain keto-acids.

Since these compounds were not resolved on the chromatogram, it was necessary to decide which one of the three compounds the mixture should be reported as. Some guidance to this problem was obtained by calculating the molar extinction coefficient for the hydrazones of the three individual keto-acids.

The molar extinction coefficient is defined by:

$D = X.l.C.$ where D = Optical Density
X = Molar extinction coefficient
l = length of light path
C = molar concentration of solute.

From the calibration curves obtained from the pure hydrazones,the value of X was calculated:

Hydrazone of:	Molar extinction coefficient, O.D.$cm.^{-1}mole.^{-1}$litre.
KIV	I7,287
KMV	I8,662
KIC	I9,375

It is apparent that there is little difference between the values presented above;it was decided to use the KMV calibration graph as the standard against which to measure the branch chain acids.

After establishing the method to be used,it was applied to selected problems,and the results of its application are reported in Part 3,below.

Part 3. Results.

PART III.

RESULTS

1. Introduction.

Studies on the blood ketoacid concentrations in several different diseases were undertaken. The values obtained in each disease are reported and discussed together below.

2. Normal subjects.

2.1. Single Samples. Blood was collected from 16 normal subjects, observing the precautions mentioned on page 34, and was processed according to the method described in Part II.

Except for M.S., A.S., J.C., P.B. and U.N., blood samples were obtained at least 4 hours after the last ingestion of food and/or drink; the five exceptions had undergone a 12 hour fast before sampling.

The individual results may be seen in Table 6, and are summarised here:

Ketoacid	Range (µg/100 ml.)	Mean (µg/100 ml).	S.D.	Coefficient of variations, %.
α-keto-glutaric	64-126	103	± 27	26
Pyruvic	300-1018	643	± 186	29
Total branch chain (as KMV)	383-853	588	±152	26

A comparison of these results, with those of other authors reported in table 5, shows that they are of the same order, but that the mean concentration of α-ketoglutaric acid is lower than that obtained by most authors studying the hydrazones by chromatography, although it is in agreement with the D.A.N.B. method of Smith and Taylor (1955).

TABLE 6.

Subject	Wt. blood (B gm.)	Wt. blood + pptant (T gm)	Vol. NH_4OH on paper (ml.)	α-ketoglutaric acid			Pyruvic acid			Branch chain acids (as KMV)		
				O.D.	O.D. blank	Concn. µg/100 ml	O.D.	O.D. blank	Concn. µg/100 ml	O.D.	O.D. blank	Concn. µg/100 ml.
M.S. (M)	5.70	36.21	0.50	0.080	0.025	84	0.348	0.124	701	0.196	0.037	853
G.W. (M)	5.02	36.43	0.50	0.057	0.007	116	0.320	0.053	820	0.100	0.029	500
T.I. (M)	5.85	36.94	0.50	0.133	0.035	118	0.501	0.133	738	0.182	0.039	762
A.S. (F)	5.15	36.55	0.50	0.073	0.023	87	0.483	0.123	812	0.186	0.056	778
B.C. (M)	5.40	36.73	0.50	0.147	0.044	168	0.630	0.163	1018	0.167	0.048	683
H.H. (M)	6.24	40.57	0.50	0.093	0.032	96	0.419	0.128	454	0.113	0.037	417
K.K. (F)	5.77	36.79	0.50	0.096	0.032	98	0.441	0.128	635	0.152	0.037	618
G.C. (M)	5.57	35.94	0.50	0.107	0.040	99	0.509	0.107	823	0.125	0.037	474
J.C. (M)	5.44	36.76	0.50	0.101	0.043	93	0.442	0.107	718	0.110	0.037	416
D.B. (F)	5.35	36.70	0.50	0.098	0.024	122	0.299	0.161	300	0.111	0.037	428
R.L. (M)	5.47	36.80	0.50	0.070	0.030	64	0.437	0.125	667	0.158	0.041	665
S.F. (M)	5.70	37.09	0.50	0.072	0.030	65	0.399	0.125	566	0.127	0.040	478
D.F. (F)	5.86	37.04	0.50	0.098	0.032	125	0.311	0.092	549	0.114	0.034	533
J.F. (F)	5.55	35.86	0.50	0.133	0.032	126	0.424	0.147	568	0.187	0.054	731
F.R. (M)	5.60	36.88	0.50	0.078	0.032	71	0.316	0.147	352	0.123	0.054	383
U.N. (F)	5.56	36.92	0.50	0.098	0.030	108	0.387	0.122	559	0.164	0.040	695

Table 6. The blood concentration of keto-acids in normal subjects.

There may be several explanations for this difference:

The quantity of α-ketoglutaric acid in blood is so small that accurate measurement of its concentration is extremely difficult. Thus, an inspection of the results in table 6 reveals that the optical density of the α-ketoglutarate blank is relatively high by comparison with that of the actual α-ketoglutarate spot. The difference between the two is very small, and a small reduction in the optical density of the blank would cause a relatively large increase in the measured blood concentration.

The primary standard in "D.N.P.H. methods" is the parent keto-acids, whose purity it is often difficult to define. This criticism is made by the advocates of enzymatic methods using lactic dehydrogenase or glutamic dehydrogenase (Hess, 1955; Seitz et al, 1955; Segal, Blair and Wyngaarden, 1956; Marks, 1961), and Segal, Blair and Wyngaarden, (1956) claim that D.P.N.H. and D.P.N., the primary standards of these methods, can be obtained in high purity, although they appear to discount the possibility that their enzyme preparation may contain small quantities of <u>other</u> enzymes using D.P.N.H. and D.P.N. as coenzymes.

As judged by the M.P. of its hydrazone, the α-ketoglutaric acid used as a primary standard in the present work was very pure, and vigorous methods of drying the hydrazone standard to constant weight were adopted. Thus the solutions of this hydrazone dissolved in NaOH to prepare the calibration graph would have a minimum unit weight:optical density ratio; in methods where less stringent drying was adopted, this ratio would be higher, and, since small quantities of the substance were being measured, this variation would cause a significant difference to the concentrations finally measured in blood. This "drying effect" would have a much

smaller influence on the final results obtained for pyruvate and branch chain ketoacids, since these are present in higher concentrations.

The method used in this study was that of de Schepper, Parmentier and Van der Haeghe (1958) with minor modifications, and inspection of their results suggests that the ranges of blood concentrations of the different ketoacids are essentially the same as those reported here. These authors did not quote mean values, but inspection of a "scatter diagram" of normal blood concentrations suggests that the mean values are very similar to those obtained in the present study.

In addition to the above observations, it will be seen that there is a wide scatter of the normal values found, the magnitude of the coefficient of variation being of the same order as those reported by Seligson and Shapiro (1952), El Hawary and Thompson (1953) and McArdle (1957). As was noted on page 36, differences between means of small samples may be due purely to sampling error.

2.2. Spontaneous Variations in blood concentration and urinary excretion.

Many papers have been published describing the blood ketoacid concentrations in fasting subjects based upon an analysis of a single blood sample, but very little has been published about possible spontaneous variations in these values over different periods of time. For this reason, it seemed advisable to obtain some indication of any possible spontaneous variation and of its magnitude.

2.2. 1. Day to day variation. In order to make the conditions at sampling both as basal and reproducible as possible, samples of blood and urine were collected from subject M.S. (a 26 year old healthy male) immediately on awakening in the morning. In this way, the effect of

exercise, which increases the blood concentration of ketoacids(see Part 1), and also has an effect on urinary production of the ketoacids(McArdle,1957) was minimised.

In the same way, since sampling took place some 12 hours after the last ingestion of food or drink, any possible dietary factors were minimised. BBlood samples were obtained on eight successive mornings, and the overnight urine on seven of these mornings.

The results are reported in tables 7 and 8. The values for the blood concentrations were calculated in the normal way, but it is necessary to make some observations about the mode of presentation of the results for urinary ketoacids.

Urine was voided on retiring to bed at 11.30 p m. and the overnight urine collected at 8.00 a.m the following morning, so that the time of collection (in all 7 samples) was 8.5 hours. The volume varied considerably, as did the concentration of the ketoacids. The effect of varying volume was avoided by estimating the quantity of ketoacid produced per hour, and the method of calculation is described in appendix 2.

From the results obtained, it is apparent that there is a considerabl day to day variation, both in blood concentrations and urinary production rates.

From the values, it does not appear that blood concentration or overnight volume influences the ketoacid production rate, and the regression coefficient calculated for production rate upon blood concentration and upon overnight volume (using the method described in Appendix 1) was NOT significant,* for both α-KG and P.

* In each case, the probability that the regression coefficient differed from zero due to chance was greater than 0·4.

TABLE 7.

Day to Day Variations in Blood Ketoacid concentrations

Day	Wt.blood (B gm)	Wt.blood + pptant (T gm)	Vol. NH_4OH on paper (ml)	α-ketoglutaric acid			Pyruvic acid			Branch chain KAs. (asKMV)		
				O.D.	O.D. blank	Concn. µg/100 ml.	O.D.	O.D. blank	Concn. µg/100 ml.	O.D.	O.D. blank	Concn. µg/100 ml.
1	6.00	37.42	0.40	0.053	0.023	56	0.297	0.099	487	0.116	0.031	552
2	6.13	37.49	0.40	0.067	0.021	79	0.411	0.101	754	0.138	0.032	632
3	4.65	35.99	0.40	0.061	0.021	77	0.386	0.095	894	0.128	0.039	727
4	5.32	36.58	0.50	0.082	0.030	86	0.428	0.122	662	0.134	0.039	609
5	4.72	36.02	0.50	0.093	0.029	115	0.454	0.125	797	0.139	0.041	606
6	5.38	35.77	0.50	0.088	0.030	93	0.435	0.124	673	0.147	0.040	611
7	5.46	36.83	0.50	0.091	0.031	99	0.311	0.124	529	0.138	0.041	558
8	5.70	36.21	0.50	0.055	0.000	84	0.348	0.123	702	0.196	0.039	853
					Mean	86 ± 17		Mean	687 ± 134		Mean	644 ± 100

TABLE 8.

Day to Day Variations of urinary α-ketoacids.

Time of collection = 8.5 hours.

Day	Vol.urine ml.	Vol.NH_4OH on paper,ml.	O.D. of * K.G.	O.D. of * P	Production rate, mg/hr.	
					KG.	P
1	245	0.20	0.468	0.450	1.04	0.53
2	247	0.20	0.411	0.452	0.92	0.44
3	450	0.20	0.288	0.374	1.22	0.76
4	265	0.20	0.480	0.706	1.19	0.90
5	280	0.10	0.232	0.295	1.20	0.79
6	215	0.20	0.492	0.483	0.97	0.50
7	425	0.10	0.132	0.137	1.02	0.61
				Means	0.93 ± 0.60	0.65 ± 0.17

* Optical densities connected for O.D. of corresponding blank.

2.2.2. Variations in blood concentrations over a short period.

The concentrations were studied over the period of 4 hours in two subjects.

The subjects were two patients in hospital for the investigation of diseases not known to have an effect on intermediary metabolism,the first for idiopathic epilepsy and the second for essential hypertension (although the first patient was eventually found to be neurotic,with a hysterical epilepsy.)

The subjects were fasted overnight (12 hours) and blood collected over the next 4 hours,whilst they rested in bed.Table 9 shows the results obtained.

The most striking overall feature is the cosiderable fall in pyruvate concentration over the 4 hour period,the concentration of the other compounds showing no consistent trend. A similar change in pyruvate was noted by Segal,Blair, and Wyngaarden(1956).

Sample No.	Time of sampling,hr.	Keto-acid	Subject 1	Subject 2.
1	0.0	KG	196	147
		P	721	600
		B.C.	616	826
2	0.5	KG	185	150
		P	716	459
		B.C.	577	990
3	1.0	KG	175	153
		P	592	510
		B.C.	493	959
4	2.0	KG	153	149
		P	520	421
		B.C.	499	773
5	3.0	KG	160	144
		P	649	382
		B.C.	490	968
6	4.0	KG	143	144
		P	446	355
		B.C.	487	653

Table 9. The levels of α-ketoacids in 2 fasting subjects over 4 hours.

All concentrations μg/100ml. blood.

3. The keto-acids in Disease.

3.I. The Adrenals.

The rather conflicting results obtained for the blood concentration of keto-acids in patients with abnormal adrenals has been noted on page 46.

Values obtained in three cases of Cushing's Syndrome and one of Addison's Disease are presented in table II.

The urinary I7-ketosteroids and I7-ketogenic steroid production rate were estimated in the routine laboratory of the Department of Clinical Chemistry of The Royal Infirmary of Edinburgh, and hydrocortisone production rate was estimated by Mr.(now Dr.) G.F.de Witt in the same department.

By measuring the rapidly falling specific activity of plasma hydrocortisone (cortisol) after administering 4-C^{I4} labelled cortisol intravenously,it was possible to estimate the turnover rate of endogenous cortisol.(De Witt,I96I)

The production rate was also estimated by studying the urine of these patients.The principal metabolite of cortisol in urine,tetrahydrocortisone, was isolated by chromatography,and from a consideration of its specific activity and the total radioactivity of the 24-hour specimen of urine,the 24 hour production rate was estimated. Only a very brief description of the method has been given here,and full details may be obtained from the Ph.D.thesis of Dr.de Witt(I96I).

Normal values found were,

from urine:8.8-I8.9 mg/24 hour.

from blood:I6.I-30.2 mg./24 hour.

The value from blood is,in fact,the turnover rate during $2\frac{1}{2}$ hours on the morning of study,extrapolated to represent a 24 hour value,whilst the value from urine estimates the total 24 hour production,and since cortisol production varies diurnally,with a maximum in the morning,the former value is higher. This normal diurnal variation is absent in Cushing's Syndrome(Forsham,I96I)

Cope and Black(I958) and De Witt(I96I) regard the cortisol production rate as the most sensitive measure of the hyper-adrenocorticism of Cushing's Syndrome.

TABLE II Blood Keto-acid Concentrations in Diseases of the Adrenals.

Patient Sex & age	Diag.	3-day I7-KS & I7-KGS prod. rate mg/24hr. KS	KGS	Fasting Bld. glucose mg/I00ml	Oral gluc. toler. curve	Cortisol prodn. rate mg/24hr. bld.	urn.	Blood keto-acid conc. µg/I00ml. α-KG	Pyruv.	Br. Chain.
M.S./F 39	C.S.	I6.9 - -	32.9 - -	70	normal	70.0	II2.0	I02	707	358
T.D./M. 38	C.S.	33.4 I9.4 37.8	- 28.6 -	I08	normal	62.4	75.8	3I	565	742
E.S./F 22	C.S.	9.8 22.3 II.0	I3.4 52.I I7.7	96	normal	-	33.8	I55	658	II40
M.R./F 53	A.D.	I.8 I.I 2.3	2.7 2.9 2.3	-	-	4.9	-	I09	462	784

Key to table

C.S.- Cushing's Syndrome.

A.D.- Addison's Disease.

I7-KS- I7-ketosteroid

I7-KGS-I7-ketogenic steroid

On the basis of these measurements, the cortisol production rate deviates markedly from normal values in all four cases, but yet no abnormality is evident in the blood keto-acid concentrations, except for that of the branch chain compounds in case 3, which is slightly above the upper limit of the normal range.

Perhaps the most interesting observation is the finding of a normal blood pyruvate concentration in all 4 cases, in keeping with the observations of Segal, Blair & Wyngaarden, (1956) and Hills, Power & Wilder(1952) who have found normal levels in patients with the same disease.

None of the cases of Cushing's Syndrome studied here showed an abnormal glucose tolerance, even though Williams(1960) quotes figures to show that 81% of patients show the disturbance. It is probably unwise to assume that the blood pyruvate and glucose concentrations are related. Thus Segal, Blair & Wyngaarden(1956) have reported two normal subjects in whom the blood glucose concentration remained constant, whilst the blood pyruvate concentration fell progressively throughout the period of the test.

The precise site of action of cortisol is not clearly understood, but Long, Katzin and Fry(1940) showed by balance studies that the liver of mice contained increased quantities of glycogen after administering cortical extract, and that glucose production from some nitrogen containing substance, probably protein, was increased. The adrenal corticoids appear to inhibit protein synthesis (Hoberman, 1950), and to increase the rate of release of amino acids from the tissues of eviscerated animals(Bondy, 1949; Ingle, Prestrud & Nezamis, 1950; Bondy, Ingle & Meaks, 1954).

C-11 oxygenated steroids appear to accelerate the degradation of amino acids (Russel, 1955).

A striking increase in glutamic-pyruvic transaminase activity has been demonstrated in the livers of rats given cortisone (Rosen, Roberts & Nicholl, 1958), although these authors regard the effect as being indirect since an increase of similar magnitude may be induced by giving a high protein diet to normal rats, and even adrenalectomised animals show a less marked, but similar response.

The observations by others of a high fasting blood pyruvate concentration in patients with Cushing's Syndrome, and of an abnormaly great rise in blood pyruvate on the administration of glucose (Frawley, 1955 ; Henneman & Bunker, 1957; Hennes, Wajchenberg, Fajans & Conn, 1957; Fajans, 1961) has been regarded as indicating a block in the utilisation of pyruvate.

In support of this theory, Frawley & Shelly, (1961) have demonstrated a diminished rate of disappearance of injected pyruvate during corticosteroid therapy. Glenn, Bowman, Bayer & Meyer(1961) observed a decreased rate of oxidation of exogenous glucose after cortisol administration in adrenalectomised rats, andindicated that the inhibiting effect occured at the pyruvate level of glucose metabolism.

Fajans(1961) infused sodium pyruvate into normal subjects before and after administering prednisone, and found that the normally ensuing rise in blood citrate was inhibited by the steroid.

Thorn, Renold & Cahill (1959) observed increased hepatic synthesis of glucose from pyruvate under influence of cortisol.

Thus it would appear that cortisol inhibits pyruvate utilisation, and the latter is thus increasingly available for the synthesis of glycogen. Perhaps the explanation lies in the observations that corticosteroids inhibit the oxidation of D.P.N.H. _in vitro_ , thus reducing the availability of D.P.N. required for the oxidative decarboxylation of pyruvate to acetyl-CoA. (Yielding, Tomkins & Munday, 1960.)

It is difficult to find an explanation for the observations of those workers who have found normal blood pyruvate concentrations in Cushing's Syndrome. No one has estimated the concentrations repeatedly in individual patients with Cushing's Syndrome; this would probably be worth doing, since Prunty(1960) has pointed out that the cortisol production rate in these patients is extremely labile, varying greatly over short periods of time. Thus the raised cortisol production rate measured in these cases can only be regarded as an indication of the hyperactivity on the day when the blood keto-acid concentration was measured, since practical problems made it impossible to study both on the same day.

An extension of the study of the keto-acids in the blood of patients with Cushing's Syndrome and with Addison's Disease would be worthwhile, in order to define with some certainty the level of branch-chain keto-acids , particularly in the latter disease; an extensive review of the literature suggests that this study has not been undertaken, probably because of the relative infrequency with which Cushing's Syndrome presents, and the rarity of Addison's Disease.

Since cortisol has been shown to promote gluconeogenesis from amino-acids, it might be assumed that the level of branch-chain ketoacids might be raised; although G.P.T. activity has been shown to be raised in rat liver treated with cortisone, there is no evidence to suggest that branch-chain amino acid transaminase activity is increased, and if the evidence discussed above is to be accepted, the effect of cortisol on pyruvic oxidase would have little effect on the branch-chain ketoacids; reference to fig.I2 shows that the end products of catabolism of these acids are acetyl-CoA or propionyl-CoA, both of which enter the citric acid cycle by routes which bypass pyruvate.Thus it would seem that the branch-chain ketoacids are unlikely to be present in abnormal concentrations in the blood of patients with hyper- or hypo- adrenocorticism.

The α-ketoglutarate concentration in these diseases has never been found to be abnormal (Hennes, Wajchenberg,Fajans & Conn,I957; Henneman& Bunker,I957).Even though G.P.T. activity is increased, the explanation for the lack of effect on the peripheral blood concentration of α-ketoglutarate may be that there is a high concentration of the compound in liver (Le Page, I950; Cavalini & Frontali, I949a), although great caution must be exercised in drawing conclusions about the changes in the tissues of a compound of great physiological importance and reactivity, when only blood concentrations have been studied.

The effect of cortisol at the pyruvic oxidase level may explain why those patients who develop "steroid diabetes" never develop ketosis, since a relatively small concentration of acetyl-CoA would be available to either enter the citric acid cycle, or to condense to form aceto-acetic acid, or the other "ketone bodies"(Forsham,I961)

3. 2. The Ketoacids in Hepatic Disease.

Fifteen patients with hepatic disease were studied, the results being presented in table 12. All these patients, with the exception of D.G., had fasted for 12 hours and rested in bed overnight.

Those concentrations outside the range of mean ± 2.S.D. which was defined in section 2.1 above are regarded as abnormal, andhave been underlined in table 12. Thus in 11 cases the α-ketoglutarate concentration is abnormal, and the pyruvate and branch chain ketoacid concentration is abnormal in only 2 cases each.

The abnormal pyruvate concentration in case D.G. is probably due to the intravenous glucose infusion which the patient was administered for some 12 hours before blood was sampled.

The finding of high concentrations of α-ketoglutaric acid confirms the observations of many groups of workers in patients with hepatocellular damage and in patients with cirrhosis (Seligson, 1952; Amatuzio and Nesbitt, 1950, Summerskill, Wolfe and Davidson, 1957; Bianchessi,1958), but an extensive review of the literature suggests that no one has previously reported abnormal concentrations of α-ketoglutarate in the blood of patients with obstructive jaundice; clearly some of these patients do present an abnormal level.

From the limited survey presented here, it would seem that a study of the blood ketoacids has no value in the differential diagnosis of hepatocellular and obstructive jaundice.

So diverse are the many activities and so great is the functional reserve capacity of the liver, that no single test can be regarded as providing an accurate, quantitative estimate of hepatic cell

Table 12.

The ketoacids in the blood of patients with Liver Disease.

Patient & age.	Diag-nosis	Ser. bilir. mg/100 ml.	Alk. phos. K-A units	Thym. turb. Units	Zn. turb. Units	G.O.T. Karmen Units	G.P.T. Karmen Units	Blood ketoacid concn., $\mu g/100$ ml.		
								α-KG	Pyruv.	Br.chain (as KMV)
W.N./32	I.H.	2.1	21	6	10	71	75	131	204	525
M.M./33	I.H.	1.5	17	12	8	137	107	187	800	670
J.S./22	I.H.	2.3	28	8	12	210	240	185	755	867
S.G./41	Cir.	0.2	8	2	4	20	20	205	804	945
S.W./57	Cir.	2.7	34	5	4	42	40	275	628	579
J.R./57	Cir.	1.1	16	12	14	105	97	142	439	795
H.C./51	Cir.	0.9	19	8	12	120	98	443	420	654
L.C./39	Cir.	0.7	26	4	5	20	20	249	688	511
J.M./51	Cir.	1.2	16	6	12	115	94	303	695	743
J.P./53	Cir.	1.5	12	5	2	20	25	342	1126	805
N.K./72	O.J.	7.1	62	3	5	50	58	263	789	834
D.G./76	O.J.	6.3	48	4	5	116	130	197	1393 *	1065
P.B./74	O.J.	7.6	63	3	4	116	93	127	509	412
H.W./56	O.J.	4.7	25	2	2	123	141	114	542	676
W.B./83	O.J.	7.0	30	1	3	50	56	180	562	769

Key. I.H. - Infective Hepatitis.
Cir. - Hepatic Cirrhosis (Subject J.R. had haemachromatosis; the remainder were idiopathic, except for L.C. and J.M., where the cirrhosis was due to alcohol.)
O.J. - Obstructive jaundice.
* This patient had been given intravenous glucose for 12 hours before the blood was sampled.
Abnormal ketoacid concentrations are underlined.

damage, and of the reserve capacity of the liver, (MacLagan, 1956; Popper and Schaffner, 1957), although the severity of parenchymatous damage is regarded as paralleling the rise in thymol turbidity and severe damage may be associated with a low serum albumin (Stewart and Dunlop, 1962). Claims have been made that the fall of esterified cholesterol in blood is related to hepatocellular damage in cirrhosis (White, Deutsch, Maddock, Downing, and Jensen, 1939) although it is now believed that this change is associated more with the degree of jaundice rather than with its cause (Zieve, 1953).

The bromsulphthalein excretion test is regarded as a sensitive index of hepatocellular damage in non-jaundiced patients, but it is so sensitive that significant retention of the dye has been observed in temporary functional disturbances such as fevers, anaemias, circulatory failure (haemorrhagic) and in rheumatoid arthritis and diabetes mellitus (MacLagan, 1956).

The most valuable indication of active hepatocellular damage is probably the demonstration of raised G-O.T. and G-P.T. activity in the serum of patients with the various hepatic diseases (Wróblewski, 1958).

The brief survey above makes it clear why a "battery" of biochemical tests are used for the assessment of liver function (MacLagan, 1956), and it was hoped at the beginning of this study that the estimation of blood ketoacids may assist in this task. Unfortunately there appears to be no evidence in the values presented here that the blood ketoacid concentration is an index of hepatocellular damage. Thus if the serum transaminase levels are accepted as a parameter of hepatocellular damage, patient J.S. has much more severe damage than patient M.M. but yet the

blood ketoacid concentrations are very similar, with equally abnormal α-ketoglutarate concentrations in both.

Similarly, in the patients with cirrhosis, although <u>active</u> cirrhosis (as indicated by an abnormally high serum transaminase activity) is associated with a high α-ketoglutarate concentration, high levels are also to be found in patients with inactive cirrhosis. These findings are in accordance with the views of Popper and Schaffner (1957) consider the blood α-ketoglutarate concentration to be "of limited diagnostic and prognostic value."

It would appear that the abnormally high blood level of α-ketoglutarate in patients with hepatic disease is due to the inability of the liver to remove and metabolise the compound efficiently. Thus the liver takes up α-ketoglutarate and pyruvic acids, as shown by a positive arteriovenous difference (Seligson, 1956,) and the blood α-ketoglutarate rose to abnormally high levels in 100% of animals subjected to porta-caval anastomosis and ligation of the hepatic arteries (Giges, Dein, Sborov, Seligson and Howard, 1953).

The precise biochemical lesion which occurs in diseased livers to cause the phenomena observed above is not known. Seligson (1956) believes that there may be some abnormality of the citric acid cycle, and in addition, he states that "in the failing liver there are suggestions that the normal reservoir of A.T.P. and other high energy phosphates has diminished".

In support of the theory that energy turnover is impaired in experimental and human hepatic injury, it has been shown that oxidation of butyrate and pyruvate by mitochondria due to a specific defect in

acetate activation. (Recant, 1956; Fischer and Recant, 1956). If the "depletion theory" of Seligson is correct, then it is surprising that only one case with cirrhosis in this series presents any abnormality in the branch chain ketoacid concentration in blood, for the catabolism of these compounds is dependent upon an adequate supply of coenzyme A, and thus indirectly upon an adequate supply of A.T.P.

Although the theory of Seligson is applicable to toxic and viral hepatocellular disease, and also, possibly, to hepatic cirrhosis, it is difficult to explain the impaired handling of α-ketoglutarate in early obstructive jaundice, where the hepatic cells were, presumably, normal before the onset of the disease. Perhaps, however, the explanation is provided by Zetterström and Ernster (1956) who showed that unconjugated bilirubin in a concentration of 20 mg/100 ml. caused uncoupling of oxidative phosphorylation by rat liver mitochondria, though conjugated bilirubin did not possess this effect.

Although this concentration of bilirubin is large and the total blood concentration of bilirubin, in both its conjugated and unconjugated form, is rarely higher than this value in obstructive jaundice, it has been shown that the concentration of both types of bilirubin is raised in the blood of patients with obstructive jaundice (Billing, 1959); thus it is quite likely that the local concentration of the pigment within and around the hepatic cells reaches the critical level found by Zetterström and Ernster. Although no quantitative estimate appears to be available for the bilirubin content in the liver of patients with obstructive jaundice, the pigmentation of that organ at post mortem is striking.

3. 3. Diabetes mellitus.

Five patients with insulin-requiring diabetes mellitus of moderate severity were studied; blood samples were taken in the early morning, after fasting overnight, and at a time when the patient was thought to be free of the effect of exogenous insulin (all were under investigation of their diabetic state, and were on a therapeutic regime of short acting insulins), and free of ketosis.

The results obtained are presented in table 13, where it will be seen that no abnormal ketoacid concentrations were found. The finding of normal blood concentrations of α-ketoglutaric and pyruvic acids is in accordance with the experience of most recent authors, although there was some earlier conflict, reviewed in part I of this thesis.

Although much work has been done on and published about the concentration of pyruvic and α-ketoglutaric acids in the blood of diabetics, there appear to have been none about the branch chain ketoacid concentrations.

The large volume by Williams (1960) testifies to the complexity of the aetiology and pathogenesis of diabetic mellitus, and it would be foolish to regard the syndrome as being purely due to deficiency of insulin secretion by the pancreas, although many of the metabolic abnormalities (e.g. nitrogen loss) may be reversed by the administration of exogenous insulin. Because of the dramatic therapeutic value of insulin in an otherwise fatal disease, it is perhaps natural that more work has been done on the metabolic effects of insulin, than on other aspects of the chemical pathology of diabetes mellitus.

The intimate effect of insulin on the metabolism of aminoacids,

is obscure, but it appears that the promotion of protein synthesis by the hormone is an indirect effect, via the stimulus to glucose metabolism, thus increasing the amount of energy necessary for the endergonic synthesis of protein. (See Fruton and Simmonds, 1958).

Certainly there appears to be no evidence that the presence or absence of insulin promotes the catabolism of aminoacids, and the author feels that it is not surprising that the branch chain ketoacid concentration in the blood of the patients studied here, is within the normal range. The intimate mechanism of the effect of insulin on carbohydrate metabolism is still not clear, and it is not proposed to discuss it here, the reader being referred to the volume edited by Williams (1960), where an exhaustive review of the problem is presented. The effect of the hormone in so far as it effects α-ketoglutaric acid and pyruvic acid has been discussed in part I.

Table 13. The blood ketoacid concentration in 5 patients with diabetes mellitus.

Patient	Age	Insulin requirements units/day.	Blood ketoacid concn. μg/100 ml. α-KG	P	Br. chain (as KMV)
M.S.	23	40	78	495	580
T.E.	32	32	107	528	420
M.H.	41	18	115	603	525
H.S.	29	46	89	508	654
P.E.	29	38	95	627	398

3. 4. The Ketoacids and Intestinal Absorption.

During the course of a study of the serum aminoacids of normal subjects and cases of "malabsorption syndrome" after the ingestion of pure casein, it was noted that the serum glutamic acid concentration did not rise, although that of other aminoacids did (Richmond and Girdwood, 1960). This phenomenon was rather puzzling, since casein contains 22.5 g. of glutamic acid per 100 g. protein (Fieser and Fieser, 1950), and the conclusion was to be drawn that glutamic acid was not absorbed, or that it was converted to some other compound. Dent and Schilling (1949) obtained experimental evidence for the absorption of L-glutamic acid in animals, and it was concluded that the glutamic acid must be converted to some other compound. Since L-glutamic acid is a highly important and active substrate of transaminase systems (see Part I) it was thought that the L-glutamic acid might be converted to α-ketoglutaric acid.

Thus it was decided to study the ketoacid concentration in the venous blood of patients to whom protein was given orally.

In the preliminary studies in this field, fasting, resting patients were given 25 g. of pure casein ("Casilan", Glaxo Laboratories Ltd.) suspended in 50 ml. of water, and blood samples taken at intervals thereafter. Each blood sample was analysed by the method described here for ketoacids, whilst serum aminoacids were assayed microbiologically using the organism Lactobacillus mesenteroides. Previous work in this field by Richmond and Girdwood (1960) had suggested that the arbitrary selection of sample timing at 0 (fasting), 0.5, 1, 2, 3 and 4 hours was satisfactory, and therefore these intervals were used here.

Table 14 shows the preliminary results which were obtained in one control and four patients with disease; the maximum level attained in the case of each ketoacid is underlined, where a rise was shown.

Unfortunately the pattern of response showed a complete lack of uniformity, and it is impossible to apply any statistical test of significance to them. When, however, the results were viewed against the background of normal, fasting subjects which was presented in section 1.3 above, it was felt that a rise in the concentration of ketoacids had occurred in some cases. Thus control M.H. showed substantial rise in all three groups of ketoacids, although the response in the other cases was small or non-existent. Subject J.R. is of particular interest since no rise in α-ketoglutarate occurred on the occasion of the first test, there was a rise when the test was repeated 3 days later; at the blood concentration exhibited by this patient, the experimental error was taken as $\pm$ 5%, and the very small rise presented in the first experiment may have been due purely to experimental error, whilst in the second experiment, the rise was greater than could be attributed to experimental error.

In the case of other ketoacids and of other patients, those rises in connentration greater than could be attributed to experimental error are underlined.

In view of the variable nature of the response described in table 14, it was decided to avoid the enzymic digestion of protein to aminoacids which occurs before absorption, in order to eliminate a possible source of an additional experimental variable. Accordingly,

Table 14. Response to 25 g. oral Casilan.

Patient & Diagnosis	Substance measured in venous blood.	0 Fasting	1 0.5 hr	2 1.0 hr	3 2.0 hr	4 3.0 hr	5 4.0 hr	Max. increase above fasting level %
M.H. Control (old Cerebral thrombosis)	α-K.G.	110	139	153	149	226	153	101
	P	438	636	782	698	831	585	90
	B.C.	676	865	1328	980	1410	1161	108
	Glutamic acid	2.95	3.08	3.12	3.06	2.92	2.85	
	Leucine	3.21	4.05	3.76	3.48	3.23	3.18	
	Lysine	5.32	4.95	5.26	5.18	4.99	4.83	
J.P. Hepatic cirrhosis	α-K.G.	327	362	330	348	337	374	14
	P.	1081	772	987	875	1295	858	20
	B.C.	752	1171	1365	-	1275	929	82
	Glutamic acid	3.2	3.1	3.1	3.3	3.1	3.2	
	Lysine	4.05	6.3	5.1	4.8	3.6	2.55	
	Phenyl-alamine	1.21	2.49	2.37	3.61	2.64	2.13	
L.C. Hepatic cirrhosis	α-K.G.	241	254	261	272	249	292	17
	P.	399	534	630	543	578	600	58
	B.C.	406	526	1290	1312	1380	1130	240
	Glutamic acid	3.52	3.48	3.60	3.41	3.38	3.52	
	Leucine	1.46	4.61	5.17	4.87	3.15	2.65	
	Phenyl-alamine	1.41	2.55	3.07	2.92	2.10	-	
J.R. Hepatic cirrhosis	α-K.G.	538	538	545	556	551	483	none
	P.	845	1139	1007	436	894	614	35
	B.C.	1183	899	1573	1431	1894	1476	33
	Glutamic acid	3.45	3.10	2.25	3.60	2.70	3.70	
	Phenyl-alamine	0.89	1.09	1.32	1.38	1.54	1.54	
	Lysine	2.54	2.86	3.20	3.00	3.30	3.50	
	Leucine	1.59	1.90	2.53	3.54	4.02	3.74	
J.R. repeated	α-K.G.	593	618	646	664	723	698	22
	P.	1291	1118	1440	1104	668	981	none
	B.C.	1041	803	1231	837	1325	1450	39
	Glutamic acid	3.10	3.00	3.20	2.60	2.90	3.20	
A.M.B. Malabsorption syndrome	α-K.G.	212	220	181	169	167	196	none
	P.	827	726	515	753	682	660	none
	B.C.	874	1193	1148	1261	1049	1102	44
	Glutamic acid	2.80	2.79	2.82	2184	281	-	
	Leucine	2.66	5.88	8.28	5.55	5.04	3.05	
	Lysine	5.30	9.00	8.50	7.30	7.20	6.40	
	Phenyl-alamine	1.09	1.92	2.87	1.92	1.54	1.39	

Keto-acids μg/100 ml blood; amino acids mg/100 ml. serum.

a further series of patients to whom pure L-glutamic acid was given were studied.

In order to maintain the "load" of glutamic acid within physiological limits, the patients were given 10 g. of the compound (equivalent to the quantity contained in approximately 50 g. of casein); the pure, free L-glutamic acid was suspended in 50 ml. water and given to the subject. Other conditions were like those of the "Casilan" series. Seven control subjects and three with "Malabsorption Syndrome" were studied, the results being presented in table 15. Again those rises in concentration which are greater than can be attributed to experimental error are underlined. It will be seen that the results are once more disappointing, for only three of the six controls and one of the three patients showed a rise in α-ketoglutaric acid concentration.

Little work on this problem of the relationship between administered glutamic acid and the blood α-ketoglutarate concentration appears to have been undertaken, although Seitz, Englhardt-Gölkel and Schaffry (1958)reported some figures.

Seitz, Englhardt-Gölkel and Schaffry report having found similarly inconsistent responses in normal subjects whose blood α-ketoglutarate concentration was measured enzymatically. Because of this variable response, interpretation of the observations presented here is difficult, although some experiments by others on experimental animals may assist in the task.

In 1949 Dent and Schilling studied the concentration of amino acids in the portal blood after ingestion of casein, and found a glutamic acid concentration lower than would be predicted from its high concentration

Table 15. Response to 10 g. L-glutamic acid orally

Patient & Diagnosis	Substance measured in venous blood	Sample 0 Fasting	1 0.5 hr	2 1.0 hr	3 2.0 hr	4 3.0 hr	5 4.0 hr	Max.rise above % fasting level.
J.C. Control	α-K.G.	242	127	189	180	183	104	none
	P.	1026	490	805	593	466	412	none
	B.C.	1778	1183	1650	1388	1213	1444	none
	Glutamic acid	2.50	2.40	2.45	2.52	2.50	2.47	
T.P. Control	α-K.G.	174	173	131	128	151	213	22
	P.	854	755	609	634	535	835	none
	B.C.	653	598	618	643	623	592	none
	Glutamic acid	2.27	2.16	2.31	2.22	2.77	2.02	
V.B. Control	α-K.G.	239	192	166	304	181	183	27
	P.	449	477	704	888	580	615	98
	B.C.	761	743	702	753	683	672	none
	Glutamic acid	1.44	1.44	1.54	1.47	1.92	1.44	
I.L. Control	α-K.G.	222	217	186	171	178	172	none
	P.	951	1082	934	756	686	587	None
	B.C.	1003	1107	799	782	755	905	none
S.M. Control	α-K.G.	89	88	87	83	81	72	none
	Glutamic acid	1.83	1.98	2.08	2.12	2.05	2.05	
B.G. Control	α-K.G.	257	281	-	294	231	258	14
	P.	643	673	-	593	531	570	none
	B.C.	912	880	-	591	682	704	none
	Glutamic acid	1.44	1.92	1.88	1.92	2.16	1.92	
U.N. Control	α-K.G.	90	109	193	129	113	-	114
A.M. Coeliac disease	α-K.G.	482	508	474	583	461	414	21
	P.	693	665	723	757	680	569	none
	B.C.	799	1089	1149	1490	1042	1158	87
	Glutamic acid	3.21	3.26	3.19	3.27	3.20	3.25	
C.M. Coeliac disease	α-K.G.	113	67	104	71	77	85	none
	P.	482	520	533	531	547	532	none
	B.C.	682	653	678	672	690	672	none
	Glutamic acid	1.92	2.21	2.16	2.4	1.92	2.16	
D.M. Sprue	α-K.G.	133	130	127	138	116	96	none
	P.	863	940	609	803	754	979	none
	B.C.	544	639	506	633	337	464	16
	Glutamic acid	3.54	3.48	3.60	3.55	3.42	3.40	

Keto-acid concentration μg./100 ml. blood ; amino acids mg/100 ml. serum.

in casein. Wiseman (1953) found that glutamic acid disappeared from the system he used for the study of its absorption by rat small intestine *in vitro*. Neame and Wiseman (1957,1958) found that the concentration of alanine in the mesenteric venous blood from a loop of small bowel containing glutamic acid was greater than that in arterial blood (dogs, cats and rabbits). This finding they regard as indicating that transamination of glutamic acid in the glutamic-pyruvic transaminase reaction, although they failed to find any change in the concentration of α-ketoglutaric and pyruvic acids after the blood had passed through the gut. Thus there is incomplete evidence that transamination occurs in the intestine of experimental animals, and this observation may explain the apparent failure to demonstrate a raised L-glutamic acid concentration in the peripheral venous blood of human subjects, if transamination occurs in the human small bowel. Unfortunately the portal blood must pass through the liver, the site of very active transaminase activity (Wróblewski, 1958) so that any L-glutamic acid in the portal blood would be rapidly transaminated and the resulting α-ketoglutaric acid rapidly metabolised by the liver. Thus any rise in the venous blood concentration of either L-glutamic acid or of α-ketoglutaric acid would probably be small, reflecting a "spill over" of these compounds when the load exceeded the reserve capacity of the liver to metabolise the compounds, and also dilution of the added compounds in the already existing "body pool" of them.

The results presented here are relatively few and are regarded merely as an indicator of a problem whose solution is still required. It has been proposed to alter the design of the experiment and to use

further techniques to attempt the elucidation of the problem (Richmond, Girdwood and Smith, 1961). The use of the sodium salt of L-glutamic acid instead of the actual free acid may be regarded as being a more sensible step, but early experience in this series of experiments suggested that human subjects found it extremely difficult to tolerate and tended to vomit the compound; perhaps the use of gastric intubation may avoid some of this effect, although Seitz, Englhardt-Gölkel and Schaffry,(1958) found equally inconsistent results when administering 25 g. of the sodium salt.

The use of a chromatographic method of estimating the aminoacids rather than a microbiological method is regarded as more accurate (Fruton and Simmonds, 1958), and in this particular series the micro-biological method has proved unsatisfactory, particularly since it failed to assay alanine, the product noted by Neame and Wiseman (1957) in their in vitro and in vivo experiments.

Finally the possibility of using C^{14} labelled glutamic acid to study the absorption and metabolism of the compound has been discussed (Richmond, Girdwood and Smith, 1961).

Since, however, this last proposal will probably necessitate animal experiment first in order to study the amount of radioactivity retained in the body and the necessary "dose" to give, the project has become of a long term nature, beyond the time allotted to this thesis.

4. General Conclusions.

The studies presented in this thesis demonstrate the diverse pathological conditions in which the keto-acids are of interest, because of their biological importance as highly active metabolic intermediates.

In addition, the inadequacy of available methods is demonstrated; as yet there is no method suitable for the separation and estimation of all known keto-acids, although a step forward has recently been made by Käser, Käser & Lestradet (1960), who managed to separate KIV-hydrazone from those of KIC and KMV. The demonstration of the presence of the "branch chain" keto-acids" by De Schepper, et al in 1958, caused "an impetus to the waning interest in keto-acids" (Smith, 1960).

Even though methods available are imperfect, the discovery of these compounds has "opened a new door" into the study of inborn errors of metabolism, with the description of "Maple Syrup disease", which was discussed in Part I. No doubt methods will be devised for the separation of the "branch chain" keto-acids which remain, as yet, unseparated, thus providing a useful means of studying the intermediary metabolism of the corresponding amino acids.

There seems to be no doubt that more keto-acids will be identified in material from human subjects. Thus, Biserte and Dassonville (1956), and McArdle (1957) have noted small traces of a hydrazone with low Rf value, but of unknown nature, and infrequent appearance, in urine. The present author has also noted occasionally a small, intensely yellow, ultra-violet opaque spot which travels immediately in front of the α-KG hydrazone on chromatograms prepared from the extracts of concentrated urine. The quantity of the unknown was so small that attempts to elute it and examine its absorption spectrum in NaOH failed, and the present author does not know its nature, but he feels that it may be a dicarboxylic acid, because of its low Rf.

The tedious, prolonged nature of paper chromatographic methods is an additional limitation in the study of clinical problems, and the introduction of a full range of rapid, specific methods comparable to the enzymatic methods available for the estimation of α-ketoglutaric acid and pyruvic acid, would be of great help.

A good deal of information is being obtained about the

role of α-ketoglutarate and pyruvate in various clinical conditions, by the application of these enzymatic methods,notably by Marks(I96I) and his colleagues, but the knowledge of the role of the "branch chain" compounds is in a most rudimentary form.

Unfortunately the clinical chemist must work with limitations not shared by his academic biochemical colleague;thus the former has only a relatively few sources of material to analyse. In the work described above in section 3.4, the aim had been to attempt to demonstrate the deamination of glutamic acid,during,or immediately after absorption,but the only material available for analysis was peripheral venous blood,the concentration of α-KG in which reflects the possible activity of the gut mucosa upon the absorbed glutamic acid,but also reflects the much greater activity of the liver.

Thus a measurement of the keto-acid content of the portal blood would have been of great help in defining the transaminase activity of the gut towards glutamic acid,much in the same way as Neame and Wiseman(I957 &I958) studied the phenomenon in experimental animals. Attempts were made to study the portal vein keto-acids in blood obtained at laparotomy,but it soon became evident that this proceedure such that it was not possible to control the conditions under which the samples were collected. Further,a surgeon worried about the technical problems of his craft cannot be distracted at intervals to collect blood.

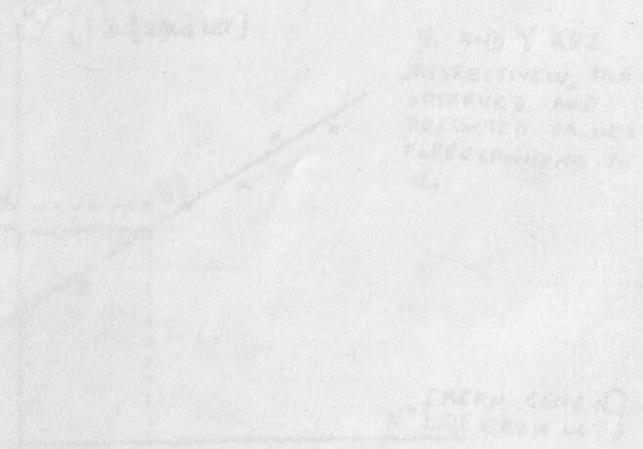
In common with many other workers,the study of keto-acids in disease presented here is rather superficial,and the author feels that much more information may be obtained by undertaking a detailed study of a few cases, with a simultaneous study of the other parameters relative to the particular disease. Thus the mystery of why some patients with Cushing's Syndrome present a high fasting blood pyruvate concentrations,whilst others do not,remains a m unsolved,and perhaps an extended study on the lines presented in Section 3.I above,with estimations related to the natural history and course of the disease,would contribute to its solution. This example,however,underlines another problem of the clinical chemist: that of obtaining a source of suitable patients,for most do not come under medical care until their disease is well developed,and many require urgent therapy because of the serious prognosis of their illness,as in this particular example(Williams,I96I).

From the few detailed studies on normal subjects presented in this work,it is evident that considerable daily,and even hourly, variation in the blood concentration of the keto-acids occurs.The author feels that an extension of this type of study,both in normal subjects and patients with various diseases may reveal further interesting information,in the same way that detailed studies e revealed the loss of normal diurnal adrenal activity in Cushing's Syndrome (Forsham,I96I;Prunty,I960) This type of project,however,requires circumstances in which it is possible to supervise patients closely, and which the clinical chemist can seldom achieve.Further,it rquires patients with sufficient courage to withstand multiple venepunctures.

On the basis of this and other studies undertaken by many workers,the diagnostic and prognostic value of blood concentrations of the keto-acids appears to be strictly limited,although the author feels that it will be necessary to undertake much more work,both on devising suitable methods and on the study of disease,before this impression is confirmed. The value of blood keto-acid concentrations in the past,appears to have been the way in which they have reflected changes in intermediary metabolism caused by some "biochemical lesions".(e.g. the demonstration of a raised concentration of pyruvic acid in the blood of beri-beri patients.) The recent discovery of "branch chain" keto-acids in blood has opened up a new field in which,already, a measurement of these compounds in both blood and urine has indicated the presence of a defect in the metabolism of amino acids in "Maple Syrup Disease."

Perhaps the most significant indication that much remains to be done lies in the words of De Schepper,Parmentier and Van der Haeghe(I958):that "the total 'branch chain' keto-acid concentration in the blood was found to be abnormal in certain diseases" (sic);the failure of the work described in this thesis to demonstrate a disease in which such an abnormality is found consistently suggests that work in this field of study must continue.

Part 4. Appendices.



APPENDIX I

Statistical formulae used and their application.

The notations used here are those of Bernstein and Weatherall (1952).

1. The calculation of Standard Deviation.

Let S signify "the sum of like terms"

n be the number of observations, $x_1, x_2, x_3, \ldots\ldots x_n$

then the mean of n terms is given by :

$$\bar{x} = \frac{S(x)}{n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 1}$$

The standard deviation(S.D.) IS calculated from the formula

$$S.D. = \sqrt{\frac{S(x-\bar{x})^2}{n-1}} \quad \text{, where } \underline{n} \text{ is less than 30. } \ldots\ldots\text{Eqn. 2}$$

$S(x - \bar{x})^2$ was computed from the convenient expression:

$$S(x - \bar{x})^2 = S(x^2) - \frac{(Sx)^2}{n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 3}$$

2. The effect of concentration on reproducibility.

For each "lot" of 4 samples from the same serum source, the mean and S.D. were calculated. The S.D. was then treated as the dependent variable ("y") with the lot mean the independent variable("x") where the two are treated graphically as ordinate and abscissa, respectively. If a straight line is drawn through a point with co-ordinates $\bar{x}, \bar{y}$, and in such a way that the sum of the squares of the deviations of individual values of y from the value, Y(that predicted from a knowledge of the equation of the line and the relevant value of x), is a minimum, then the gradient of

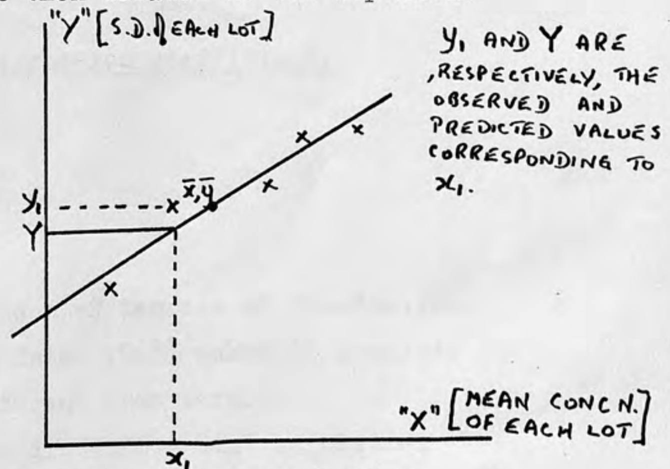


Fig. 29 The regression line.

the line may be called the "coefficient of regression", given by the equation:

$$b = \frac{S(xy - \bar{x}\bar{y})}{S(x - \bar{x})^2} \quad \text{...............Equation 4}$$

$S(xy - \bar{x}\bar{y})$ is calculated from the equation:

$$S(xy - \bar{x}\bar{y}) = S(xy) - \frac{S(x).S(y)}{n} \quad \text{........Equation 5}$$

Standard Error of regression coefficient.

The sum of squares of deviations of values of y from the predicted value, Y, is given by

$$S(y - Y)^2 = S(y - \bar{y})^2 - b.S(xy - \bar{x}\bar{y})$$

therefore the variance of this value

$$s^2 = \frac{S(y - Y)^2}{\text{number of degrees of freedom}} = \frac{S(y - Y)^2}{n - 2}$$

$$\text{S.E. of } b = \sqrt{\frac{s^2}{S(x - \bar{x})^2}}$$

The statistical significance of the regression coefficient.

The t value is obtained:

$$t = \frac{b}{\text{S.E. of } b}$$

With the value of t thus obtained, and n -2 degrees of freedom, then reference to the tables of Fisher and Yates(1948) makes it possible to define the probability of b being different from zero.

It is hoped that the above discussion will be clearer after the relevant figures have been calculated for the keto-acids, below.

Since all samples of serum were treated similarly, the results presented below are the optical densities of the final eluates, each multiplied by IO^3, but the lowest and highest values are converted to concentrations in the relevant samples of serum, and were reported in Part 2 above.

a) Pyruvic acid.

Each figure below is the sum of the individual values for the two isomeric spots, P_I and P_2.

	"Lot"						
Sample	A	B	C	D	E	F	
I	43I	350	52I	608	844	550	
2	426	329	489	582	879	552	
3	429	3I8	5I0	668	797	6I2	
4	435	353	582	65I	775	6I8	
	430.25	337.50	525.50	627.25	823.75	583.00	Lot means("x")
	3.82	I6.87	39.94	39.34	46.74	37.04	S.D. for each Lot ("y")

$n = 6$

$S(x) = 3327.25 \quad S(y) = I83.75.$

$S(x^2) = I,987,067.I875 \quad \frac{(Sx)^2}{n} = I,845,098.7604 \quad S(x - \bar{x})^2 = I4I,968.427I$

$S(y^2) = 6,998.6I77 \quad \frac{(Sy)^2}{n} = 5,627.3438 \quad S(y - \bar{y})^2 = I,37I.2739$

$S(xy) = II3,058.7200 - \frac{Sx.Sy}{n} = I0I,897.03I \quad S(xy - \bar{x}\bar{y}) = II,I6I.689$

$$b = \frac{S(xy - \bar{x}\bar{y})}{S(x - \bar{x})^2} = \underline{0.07862I}$$

$$b.S(xy - \bar{x}\bar{y}) = \underline{877.5423}$$

$$S(y - Y)^2 = I,37I.2739 - 877.5423 = \underline{493.73I6.}$$

$$s^2 = \frac{493.73I6}{n - 2} = \frac{493.73I6}{4} = \underline{I23.4329.}$$

$$\text{S.E. of } b = \sqrt{\frac{I23.4329}{I4I,968.427I}} = \underline{\pm 0.029486.}$$

$$t = \frac{0.0786}{0.0295} = \underline{2.664.}$$

With this value of t, and 4 degrees of freedom,

$$\underline{0.1 > P > 0.05.}$$

α-ketoglutaric acid.

	"LOT"						
Sample	A	B	C	D	E	F	
I	I53	I26	248	202	I89	428	
2	I55	I32	252	208	I98	398	
3	I48	I34	254	I96	208	429	
4	I49	I34	248	I93	205	422	
	I5I.25	I3I.50	250.50	I99.75	200.00	4I9.25	Lot means ("x")
	3.30	3.79	3.00	6.65	8.45	I4.49	S.D. for each Lot ("y")

$n = 6$

$S(x) = 1,352.25$ $S(y) = 39.68$

$S(x^2) = 358,589.6875$ $\frac{(Sx)^2}{n} = 304,763.3875$ $S(x - \bar{x})^2 = 53,826.3000$

$S(y^2) = 359.7392$ $\frac{(Sy)^2}{n} = 262.4171$ $S(y - \bar{y})^2 = 97.3221$

$S(xy) = 10,842.28$ $\frac{Sx.Sy}{n} = 8,942.88$ $S(xy - \bar{x}\bar{y}) = 1,899.4000$

$$\underline{b = 0.0443875.}$$

$$b.S(xy - \bar{x}\bar{y}) = \underline{84.3096175}$$

$$S(y - Y)^2 = \underline{13.0125.}$$

$$\underline{s^2 = 3.253125}$$

$$\text{S.E. of } b = \sqrt{\frac{3.253125}{53,826.3000}} = \underline{\pm 0.00777}$$

$t = \frac{0.04439}{0.00777} = 5.71.$ With 4 degrees of freedom, $\underline{\underline{P < 0.01}}$

c) Branch chain keto-acids.

	"LOT"				
Sample	A	B	C	D	
I	389	443	30I	473	
2	362	4I5	326	45I	
3	40I	432	330	437	
4	390	45I	3I5	46I	
	385.50	435.25	3I8.00	455.50	Lot means ("x")
	I7.58	I5.59	I2.99	I4.25	S.D. of each lot.("y")

$n = 4$

$S(x) = I,594.25$ $\quad S(y) = 60.4I$

$S(x^2) = 646,657.0625 \qquad \frac{(Sx)^2}{n} = 635,408.2656 \qquad S(x - \bar{x})^2 = II,248.7969$

$S(y^2) = 9I9.2925 \qquad \frac{(Sy)^2}{n} = 9I2.342 \qquad S(y - \bar{y})^2 = 6.9505$

$S(xy) = 24,255.7075 \qquad \frac{Sx.Sy}{n} = 24,077.I606 \qquad S(xy - \bar{x}\bar{y})^2 = I78.5469$

$$b = \frac{I78.5469}{II,248.7969} = \underline{0.0I58725.}$$

$$b.S(xy - \bar{x}\bar{y}) = \underline{2.83399}$$

$$S(y - Y)^2 = 6.9505 - 2.83399 = \underline{4.II64}$$

$$s^2 = \frac{4.II64}{n - 2} = \underline{2.0582.}$$

$$\text{S.E. of } b = \sqrt{\frac{2.0582}{II,248.7969}} = \underline{\pm 0.0I353}$$

t value $= \frac{0.0I58725}{0.0I353} = \underline{I.II7574.}$ With 2 degrees of freedom,

I.II&£

$$\underline{\underline{0.4 > P > 0.3}}$$

Appendix 2.

The formulae used to calculate Concentrations in Biological Fluids.

Let O.D. = Optical density of NaOH extract from chromatogram, against corresponding blank.

X = Concentration of hydrazone in the NaOH extract (mg./100ml.)

m = Gradient of line of established calibration curve.

A = Volume of blood, urine, or serum taken.

B = Total volume of blood, urine, or serum plus precipitant.

C = Volume of protein free supernatant taken to react with 2,4-DNPH.

D = Vol. of ammoniacal extract applied to the chromatography paper.

E = Vol. NaOH used to elute the individual spots.

Since the straight line of the calibration graphs pass through the origin, the equation for the line is:

$$O.D. = m.X.,$$

therefore $X = \frac{O.D.}{m}$ ....Equation I.

but E ml. NaOH were used to elute the spot, therefore the total quantity of hydrazone present in the alkaline solution is:

$$\frac{O.D.}{m} \times \frac{E}{100}.$$

This quantity was obtained from D ml. of ammoniacal solution, and therefore the quantity of hydrazone present in 1.0 ml. ammoniacal solution is : $\frac{O.D}{m} \times \frac{E}{100} \times \frac{1}{D}.$

This quantity represents the keto-acids obtained from an aliquot, C, of the total B ml. of deproteinised solution.

Therefore the total quantity of keto-acid obtained from the source is: $\frac{O.D}{m} \times \frac{E}{100} \times \frac{1}{D} \times \frac{B}{C}$ (AS THE HYDRAZONE)

And the volume of the original source (blood, serum, or urine) was A ml. therefore the concentration in the original source is:

$$\frac{O.D.}{m} \times \frac{E}{100} \times \frac{1}{D} \times \frac{B}{C} \times \frac{100}{A}$$ mg. hydrazone/100ml. ....Equation 2.

Before the formula derived in equation 2 can be applied, however, it is necessary to apply corrections:

1. To obtain the concentration in terms of the free acid, the above expression must be multiplied by the factor:

$$\frac{\text{Mol.Wt. of free acid.}}{\text{Mol.Wt. of hydrazone.}}$$

2. Corrections for losses during extraction and chromatography must be applied. The corrections, based upon estimates obtained in Part I, are presented in tabular form below.

Keto-acid.	Value of "m" O.D.units/mg.	Mol.Wt. Correction for free acid.	Correction for losses.
α-KG.	0.52	$\frac{146}{328}$	$\frac{100}{75}$
Pyruv.	0.77	$\frac{88}{270}$	$\frac{100}{70}$
KMV.	0.60	$\frac{130}{312}$	$\frac{100}{60}$

In calculating the concentration of any keto-acid, the relevant value for "m" is inserted in equation 2, and the formula thus obtained multiplied by the corrections for recovery and for the conversion to "free" keto-acid.

The formula derived in equation 2 is satisfactory for application to circumstances where the quantities of blood, serum or urine are measured by volume, but, as has been seen above, in many estimations these quantities were measured by weight, and a small correction must be applied :

the specific gravity of blood is taken as 1.060 (Marks, 1961).

Let b = weight of blood, in gm.

and T = weight of blood plus precipitant.

Then the volume of blood is: $\frac{b}{1.060}$

and the volume of blood plus precipitant is:

$$(T - b) + \frac{b}{1.060}$$

thus, substituting, equation 2 becomes:

$$\text{concentration} = \frac{\text{O.D.}}{m} \times \frac{E}{100} \times \frac{I}{D} \times \frac{100}{C} \times \left[\frac{1.060T - 0.060b}{b}\right]$$

This correction produces final blood concentrations approximately 5% higher than those obtained by using equation 2,and ignoring the slight error due to the differing S.G. values of blood and water.

An equation similar to that in equation 2 was derived by De Schepper,Parmentier and Van der Haeghe(I958c)

The production rate of urinary keto-acids.

Production rates of the urinary keto-acids were calculated by multiplying equation 2 by the factor:

$$\frac{\text{Volume o/n urine}}{100} \times \frac{1}{\text{Time of collection(hr.)}}$$

after the equation was corrected for recovery and for conversion of the result to the "free" acid.

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