S U M M A R Y.

BILE ACID METABOLISM WITH SPECIAL REFERENCE TO THE INTRAVENOUS CHOLIC ACID TOLERANCE TEST OF LIVER FUNCTION.

1. Historical.

The chemistry of the bile acids and their relationship to some other steroids is briefly reviewed. The nature of conjugation, the biological splitting of conjugated bile acids, and the action of bacteria on bile acids is discussed. The physiological importance of bile salts is briefly indicated, and it is suggested that the distribution of the different bile acids in the various animal species has an evolutionary significance. Animal experiments on bile acid metabolism and the effects of oral and intravenous administration of bile salts in animals and man are described. An account of the analytical methods which have been used in the determination of bile salts in body fluids is given.

2. Experimental.

The colour reaction used here in the estimation of bile salts is described in detail, with recovery of added cholates from blood and the accuracy to be expected.

Blood cholate levels in subjects without liver disease, show little diurnal variation, but some alteration over a period of months. Significant differences are found both between the blood cholate levels in subjects with and without

liver disease, and in different types of jaundice. There is, however, considerable scatter, so that the estimation is of little diagnostic value. The intravenous cholic acid tolerance test of liver function is compared with other liver function tests, the general biochemical findings, and the histological appearance of the liver. The results of the test in normal subjects, and in obstructive jaundice, active and latent cirrhosis, acute hepatitis, secondary malignant liver disease, and haemolytic jaundice are discussed. The intravenous cholic acid tolerance test has proved insensitive as a liver function test and unsuitable for routine clinical use, but interesting information about the metabolism of bile salts in liver disease has been obtained.

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BILE ACID METABOLISM

with special reference to the

INTRAVENOUS CHOLIC ACID TOLERANCE

TEST OF LIVER FUNCTION

A thesis for the degree of M.Sc. in Biochemistry

By

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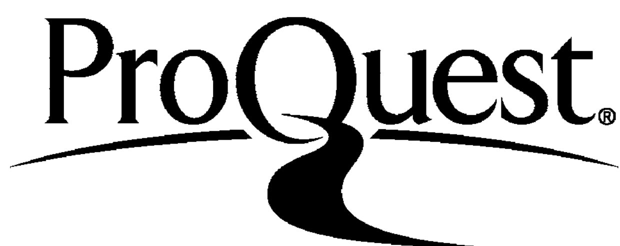

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BILE ACID METABOLISM, with special reference to

the INTRAVENOUS CHOLIC ACID TOLERANCE TEST OF LIVER FUNCTION

1. THEORETICAL

1) Fundamental chemistry of the bile acids.

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5) Action of bacteria on bile acids.

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Group D, Acute Hepatitis
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4 4) The intravenous cholic acid tolerance test of liver function.

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Group B. Obstructive Jaundice.
Group C. Cirrhosis.
Group D. Acute hepatitis.
Group E. Secondary malignant disease.
Group F. Haemolytic jaundice.

7) Discussion.

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1. THEORETICAL.

1. A BRIEF ACCOUNT OF SOME OF THE FUNDAMENTAL CHEMISTRY OF THE BILE ACIDS.

The bile acids are steroids, monobasic hydroxylated carboxylic acids, derived from the hydrocarbon cholane.

Cholane

The naturally occurring bile or cholic acids are mono-, di-, or trihydroxy cholanic acids.

Side Chain

"R" = CH_3

$CH-(CH_2)_2$ COOH.

Cholanic acid.

The formulae of the commoner bile acids are shown in the following Table (1).

The Structural formulae of these compounds. The present day formula for the ring structure of the steroids was arrived at in 1932. It is impossible to give all the evidence here, but much of the earlier work was done by Wieland, Windaus and their co-workers.[1] Diels & Karstens,[2] (1930) investigated the dehydrogenation with selenium at high

TABLE 1.

THE COMMONER BILE ACIDS.

1.	CHOLIC	3:7:12: trihydroxycholanic
2.	DEOXYCHOLIC	3:12: dihydroxycholanic
3.	CHENODEOXYCHOLIC	3:7: dihydroxycholanic
4.	LITHOCHOLIC	3: hydroxycholanic
5.	URSODEOXYCHOLIC	3:7: dihydroxycholanic
6.	HYODEOXYCHOLIC	3:6: dihydroxycholanic

temperatures, of various steroids. From cholic acid, cholatrienic acid and cholesterol, he obtained, among other products, the same aromatic, polycylic hydrocarbon chrysene.

Chrysene

Dehydrogenation with selenium also gave a hydrocarbon later identified by Cook & Hewett[3] (1933) as 3 methylcyclopentanophenanthrene.

CH_3 17 16 15

17 - Methylcyclopentanophenanthrene

As a result of intensive structural research into both the ring system and the side chain, Rosenheim and King[4] (1932)

TABLE 2.

THE CHOLANIC ACIDS.

Acid	M.P.	Source
Cholanic	168°	Coprostane, Cholic, Deoxycholic, Chenodeoxycholic, Lithocholic, Ursodeoxycholic.
*Allo*cholanic	170°	Cholestane, Hyodeoxycholic, Scillaridin A.
Bufocholanic	236°	Bufodeoxycholic.
Iso bufocholanic	179°	Bufotalin.

proposed the cyclopentanophenathrene structure for the steroids, and this formula was immediately accepted. The previous attempts at a satisfactory structure by Wieland, Windaus, and others, did not allow for 2 carbon atoms and the X-ray findings of Bernal,[5] (1932) agreed with the new formula. Cholanic acid, the parent substance of the bile acids can be obtained from them by removal of the hydroxyl groups, (Table 2). Allo-cholanic acid, the C_5 stereoisomer is the parent substance of hyodeoxycholic acid. Dehydrating agents give unsaturated acids. The relation of cholic acid to deoxycholic can be shown in this way (Boedecker & Volk,[6] 1922)

Cholic Acid

e.g. $ZnCl_2$

3:12: Dihydroxycholenic acid (Callow's[7] (1936) formula)

inter alia

[H]

Deoxycholic

Oxidation of the hydroxyl groups in the bile acid molecule, gives rise to Keto acids. Cold chromic acid on cholic acid, gives the triketo acid, dehydrocholic. (Hammarsten,[8] 1881)

Dehydročholic acid

3:7:12 Triketocholanic

Partial reduction of the triketo acid gives hydroxyketoacids and similarly, partial oxidation of cholic acid gives hydroxyketo derivatives. (Kazivo & Shimada,[9] 1937: Gallagher & Long,[10] 1943: Haslewood,[11] 1942)

ISOMERISM

A full account of the stereochemistry is given by Sobotka[12] (1937) and Fieser[13] (1936). Since there are 8 asymmetric atoms in the cholane molecule, the theoretically possible number of isomers is 2^8:256. However, among the naturally occurring steroids, only very few isomers have as yet been identified.

18 17 13 14 10 9 8 5

Centres of asymmetry in
Cholane molecule.

The isomerism of cholesterol - Windaus[14] & Staden (1915), Ruzicka[15] (1933) prepared and investigated the four theoretical hydroxyl compounds, cholestanol, epi-cholestanol, coprostanol and epi-coprostanol. These compounds are identical except for the spatial arrangement of the hydrogen and hydroxyl groups at C_3 and the relation of the hydrogen at C_5 to the methyl group at C_{10}.

CH_3 HO H I II H

Cholestanol

CH_3 H OH I II H

Epi-cholestanol

Transfusion of rings
I & II

CH_3 HO H I II H

Coprostanol

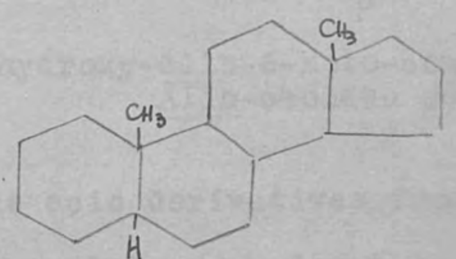
CH_3 H HO I II H

Epi-coprostanol

Cis-fusion of rings I & II

The configuration of these compounds was largely determined by Ruzicka[16] (1934). The bile acids, scymnol and coprosterol are coprostane derivatives, possessing <u>cis</u> fusion. The differentiation between <u>cis</u> and <u>trans</u> fusion can be shown by the degradation products in each case, since <u>cis</u> compounds give aetiocholanic acid, whereas <u>trans</u> compounds give aetio-<u>allo</u>cholanic acid. Further degradation gives the corresponding cholanes.

Aetiocholanic acid



Aetio-cholane

Aetio-<u>allo</u>-cholanic acid

Aetio-<u>allo</u>-cholane

The relation of these substances to decalin, (decahydronapthalene) $C_{10}H_{18}$ which also has <u>cis-trans</u> isomerism, was shown by Windaus[17] (1926): Windaus, Huechel & Revery[18] (1923) and Ruzicka[19] (1933)

The change over from the cholane to the <u>allo</u>-cholane series can be illustrated by 3:6 bile acids and derivatives, which undergo a stereochemical inversion - Hyodeoxycholic acid is converted to the <u>allo</u>-acid. (Windaus, 1923)[20]

3-hydroxy-6-keto-compound
Cholane series

3-hydroxy-allo-6-keto-compound
<u>Allo</u>-cholane series

The configuration of some of the bile acid derivatives from amphibian biles have not yet been adequately worked out. Possibly steric isomers as yet unknown in nature may be found.

The relationship of cholic acid to cholesterol, coprostanol and ergosterol can be shown by comparing the degradation products. (Table 3.)

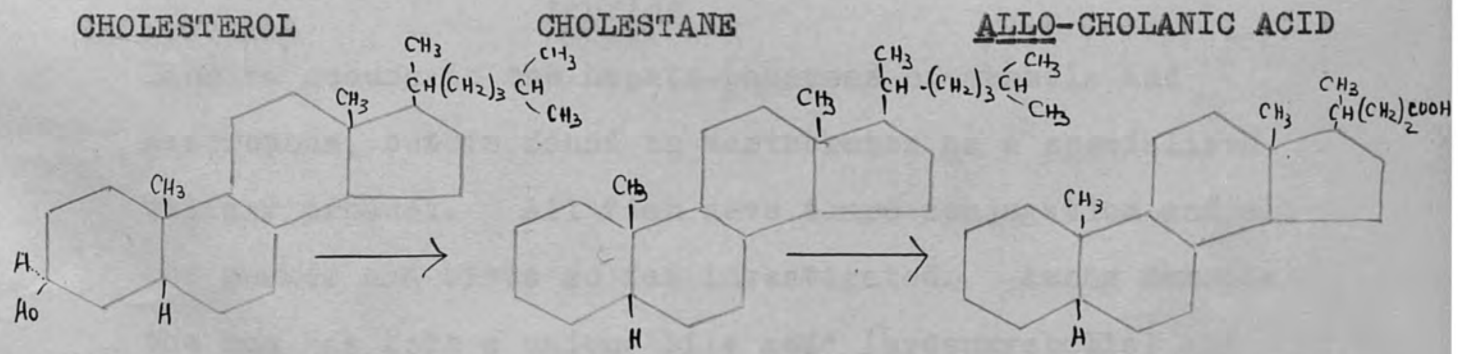
It is both interesting and significant that no intermediates from natural catabolism of the tetracyclic ring system have yet been isolated. Probably such compounds exist, but are too labile for detection by the yet available methods.

TABLE 3.

RELATIONSHIP OF CHOLIC ACID TO PLANT AND ANIMAL STEROLS AS SHOWN BY SOME OF THE DEGRADATION PRODUCTS.

CHOLIC ACID → CHOLANIC ACID

CHOLESTEROL → CHOLESTANE → ALLO-CHOLANIC ACID



COPROSTEROL → COPROSTANE → CHOLANIC ACID

ERGOSTEROL → ERGOSTANE → BIS NORCHOLANIC ACID

I.

2. CONJUGATION

a) The conjugated bile acids are glyco and taurocholic acid. The bile and molecule is joined with the amino acid, through a peptide link.

Conjugation greatly increases solubility in water. The hydrocarbon part of the molecule makes these substances soluble in fat solvents, and the three hydroxyl groups and the amino-acid residue, allow solution in water.

Glycocholic acid
or-CO-$NHCH_2So_3H$
taurine

Taurine occurs in the hepato-pancreas of mussels and gastropods, but is found in vertebrates as a specialised biliary product. All fish have tauro-conjugation and all the snakes and birds so far investigated. Among mammals the hog has both a unique bile acid (hydeoxycholic) and very little taurine in its bile. Glyco-conjugation predominates over tauro in many herbivores such as the ox, kangaroo, musk ox and hippopotamus, but in the tauro group which contains all the carnivores, the guinea pig, sheep and goat must be included. The process by which the bile acids are conjugated with amino-acids is analagous to the detoxification mechanism by which foreign and possibly harmful substances are eliminated from the body - i.e.

the conjugation of benzoic acid with glycine and subsequent elimination from the body as hippuric acid. This reaction occurs in both liver and kidney, and cholic acid conjugation also occurs in the liver. According to Whipple[21] (1919) administered cholic acid is conjugated in proportion to the available amount of amino acids. Josephson, Jungner & Rydin[22] (1938) gave 250 mg. cholic acid to cats, which was followed by an enormous excretion of free bile acid in the first 30 minutes, after which the excretion of conjugated acids increased. In 1939, Josephson, Jungner, & Lawson[23], found this was also true for humans, and they regarded this as evidence for the enzymatic nature of conjugation. On the other hand the time lag may just be necessary for the mobilisation of the required amino-acid. Since the ability of the liver to conjugate extraneous substances with amino acids is disturbed under pathological conditions, it is reasonable to suppose that this loss of function extends to the liver's own secretion. An increase in free bile acids in liver disease might, therefore, be expected. This was confirmed by Schoenheimer and Andrewes[24] (1932) and Collip & Doubilet[25] (1936). In dogs where conjugation is 100% tauro, if glycocholic acid is introduced into the intestine, 25 to 30% glycocholic appears in the fistula bile. Similarly, guinea pigs, whose bile is devoid of cholic acid, when fed ox bile, secrete a Pettenkoffer

positive bile. These experiments (Weiss, (1884)) were repeated by various workers and proved both resorption of conjugated acids and their excretion unchanged in the bile. Hydrolysis and then re-conjugation in the liver, therefore, does not occur. The reverse experiment-administering taurocholic acid to the hog has not apparently been tried.

Taurine & Sulphur Metabolism The metabolism of taurocholic acid in dogs has recently been fully investigated by Virtue and Doster-Virtue[27] (1940). The results can be tabulated:-

Table 4.

The Metabolism of some Sulphur compounds, in Dogs.

Compound	Effect
1. Cystine	No increase in taurocholic acid
2. Cholic acid	Slight increase " " "
3. Cystine & cholic acid	Marked increase " " "
4. Cystomine	No increase in " " "
5. Thioglycollic acid	" " " " " "
6. Cystine Disulfoxide	Increase
7. Cysteine sulfuric acid	"
8. Cysteic acid	"
9. Cysteine	"
10. Homocysteine	"

If cholic acid alone is given for a considerable period, the resulting increase in taurocholic is not maintained. This indicates that the animal can cope with the conjugation of the

amount of bile acids normally available, but if this is greatly increased or the amino-acid supply decreased, as by fasting, then free bile acids will be excreted. Cystamine and thioglycollic acid are obviously not metabolised by the dog unlike the other sulphur compounds tried. Under normal conditions enough taurine is available for conjugation, so that the effect of administering excess amino-acids or protein, only increases the biliary output when the taurine reserves are depleted, or when extra bile acids are also supplied. Normally, therefore, an excess of amino acids merely increases the renal output of neutral sulphur. Cystinuria has not yet been related to biliary sulphur metabolism. Cholic acid fed to cystinuric subjects had no effect in reducing the cystinuria. Epinger[28] (1923) reported a possibly significant difference from the normal in the G.T. ratio of such a case. Further investigation of this field is needed.

Ratio of

Glycocholic: Taurocholic in the bile	Cystinuric subject	8.75: 1
" " " "	Control 1)	2.07: 1
	Control 2)	1.0: 1

Rosenthal and Falkenhausen[29] (1923) concluded from the available data, that taurine is the amino-acid of preference, only the excess cholic acid being combined with glycine. The G.T. ratio is thus regulated by the existing amounts of glycine, taurine and cholic acid.

In pernicious anaemia where abnormally large amounts of taurine are made available by protein breakdown, taurocholic acid only is produced. Jacubowitsch[30] (1886) reported high taurocholic acid levels in children's bile, which is plausible physiologically, but the analyses have been queried.

2.

b) BIOLOGICAL SPLITTING OF CONJUGATED BILE-ACIDS

1) Enzymic action. The power of splitting conjugated bile acids has been attributed to many enzymes. Smorodinev[31] (1923) found that extracts of horse and dog kidney, rich in 'histozyme' could also hydrolyse glyco and taurocholic acids. Preparations of dog liver contained an enzyme which hydrolysed tauro but not glycocholic. Josephson[32] (1933) also reported species specificity since extracts of dog and cat pancreas acted more strongly on tauro than glycocholic acid; whereas pancreatic extracts from two herbivorous animals, the ox and the horse, had hardly any action on taurocholic acid. Karasawa[33] (1926) reported cleavage of glycocholic acid by testis extract.

Negative results were obtained with liver pulp by Domeninco[34] (1926). Rosenthal[35] (1927) after incubating taurocholic acid with human liver and spleen pulp for 5-6 days, found evidence of hydrolysis. Grassmann, and Basu,[36] (1936) confirmed Smorodinev's observation, but the cleavage only occurred to a limited degree. At pH 8.0 the liver extracts they used, produced no hydrolysis. Mazza and Stolfi,[37] (1932) claimed to have found a specific enzyme with a reversible action, capable of hydrolysing conjugated bile acids and of building up glyco and taurocholic acids from the bile and amino acids.

This enzyme was present in liver extracts from various animals and the optimum pH was 6.0. So Grassmann's negative results were explained. The action of their enzyme on hippuric acid was not mentioned.

The unsatisfactory position was partly cleared up by Fraenkel[38] (1936). He showed that liver contained an enzyme 'Histozyme' active over a pH range of 7 - 8 which was capable of splitting hippuric acid but not bile acids. This differed from the kidney enzyme which could hydrolyse glyco and taurocholic acids. Even over a wide pH range he failed to find any indication of a liver enzyme such as Mazza reported.

In spite of this, in 1939, Takahashi[39] claimed to have isolated 'glyco and Taurocholase' from kidney, liver and skeletal muscle, and to have found small amounts in other tissues. The richest source was dog kidney, but rat liver and rabbit, kidney and liver also contained it in considerable amounts. The optimum pH was 7-9.0, but activity was manifested over a range of 3.0 - 11.0. They assumed this to be identical with 'Histozyme' but did not mention its action on hippuric acid. The physiological importance of such an enzyme in kidney, is negligible, since except in severe jaundice, bile acids do not pass through that organ. However, such an enzyme, having both the building up and hydrolysing action, might be expected in liver. Josephson and Larrson[23] (1939) regarded the delay before

the excretion of conjugated bile acids after the injection of considerable quantities of cholic acid, as evidence for the enzymatic nature of conjugation., There is obviously a need for less theory and more experimental facts with regard to the question.

2) Bacterial cleavage Mylius[40] (1886) reported that the steroid portion of the bile and molecule was changed under bacterial influence. Licht[41] (1924) differentiated between bacterial destruction of bile acids in the intestine and parenteral decomposition by enzymes of parenchymatous origin. Rosenthal[35] (1927) found no evidence of bacterial attack at the peptide link, and suggested that decomposition might occur elsewhere in the molecule. Kashiro[42] (1925) held the theory that side chain degradation occurred. Grossmann and Basu[36] (1931) working with faecal infusions, pure cultures and putrefying bile, obtained positive evidence of bacterial destruction. Salkowski[43] (1917) found no conjugated bile acids in cadaver bile which had autolysed under aseptic conditions in the presence of chloroform. Aschner[44] (1935) isolated bacteria from soil, human and dog intestines, and human faeces, which grew on synthetic nutrient media containing glyco and taurocholic acids as the sole source of carbon and hydrogen. These acids were split by the bacteria into steroid and amino acid. The organisms

were Gm - ve, motile and produced thick membranes and pigments in media containing bile acids, but did not grow in vitro at 37°C. At this temperature they were capable, however, of hydrolysing the conjugated acids. These bacteria were not found in every specimen of faeces examined.

I.

3. PHYLOGENETIC DISTRIBUTION OF THE BILE ACIDS AND POSSIBLE SIGNIFICANCE

1) In Elasmobranch Fish: Scymnol

Bile acids have not yet been found in invertebrates, when reliable analytical methods were used. A steroid similar to the bile acids was found in the elasmobranchs. Table 5. Scymnol was isolated from shark and dogfish bile (Hammarsten,[45] 1898), and is probably a phylogenetic precursor of the bile acids.

Scymnol $C_{27}H_{46}O_5$

Scymnol is a tetrahydroxyoxide, a polyvalent alcohol derived from coprostane, possessing cis fusion of rings AB and giving the same colour reactions as cholic acid. The relationship between scymnol and the bile acids was proved by Tschesche[46] (1932) who obtained 7:12 - diketo-cholanic acid from scymnol. The side chain in scymnol consists of 8 carbon atoms with an ethylene oxide linkage. The structure was partly elucidated by Windaus[47] (1930)

7:12-diketocholanic acid

TABLE 5.

BILE ACIDS IN 1) FISH.

Fish	Bile acid	Type of conjugation.		Author	Year	Ref.
A. ELASMOBRANCHS.						
Dogfish	(			Hammarsten	1898	45
Tiger Shark	(Scymnol	Sulphuric acid ester	also 3-7-12 24-25 cholestone.	Ohta	1937	53
Haifi-fish	(					
B. TELEOSTS.						
Herring	(	T		Scherer	1856	54
Sheat fish	(Unspecified	T		Schlossberger	1859	55
Bellone	(	T		Otto	1868	56
Globe fish (Tetradon)	Cholic	T		Teraoka	1927	57
Moray	"	T		Hosokawa	1927	58
Sweetfish (Plecoglossus)	"	T	Entirely vegetarian diet (Moss)	Kobayashi	1927	59
Amberfish (Seriola)	"	T		Makino	1934	60
Gadus macrocephalus tilesius.	"	T		Tsuji & Higashi	1938	61
Goldfish	"	T		Okamura & Hatakayama	1928	62
Anago	"	T		"	"	"
Salmo (milkschish Walbaum)	"			Fukui	1937	63
Tunny	"			Shimada	1937	64
Swordfish (Xiphius)	"			Tsuji & Higashi	1938	61
Mullet (Mugil)	Cholic, Chonodeoxycholic	Unconjugated.		Watanabe & Niyazi	1935 1937	65 66
Haddock (Japanese)	" "			Ishihara	1938	67
Sebastodes inermis	" "			Ashikara, Kim & Sihn	1938	68
Trigger fish (Monacunthus).	" "			"	"	"

P.T.O.

TABLE 5 (CONT'D).

Fish	Bile acid	Type of conjugation		Author	Year	Ref.
B. TELEOSTS (CONTINUED).						
Anchovy (Japanese)	Cholic, Chonodeoxycholic			Talenti	1937	69
Paralicthys olivaceous	" "			Sihn & Kim	1938	70
Euthynnus	"			"	1939	71
Conger eel	"			Takahashi & Mori	1940	72
Sparus macrocephalus	" "	T		Hosegawa, Tukamoto & Kataoka	1940	73
Ancanthrogobins	" "					
Perophthalorus cantonensis	" "	Tetrahydroxy-norstero cholanic acid.	Position of hydroxyl groups uncertain.	Mabuti, H.	1901	74
Inimicus Japonicus Cuvier et Valencinnes.	" "			Ohta	1939	75
Inimicus	" "			Isaka & Azato.	1940	76
Gigi	"					
Bari & Fugu fish.						

and finally by Askikari[48] (1939). He oxidised and then hydrolysed the tetraacetate, and obtained cholic acid. The hydroxyl group in ring A was therefore at C_3 and not C_4 as had been thought. This was confirmed by Bergmann and Pace[49] (1943). Scymnol is also present in the bile of the blue skate and grey dog-fish (Cook,[50] 1941) but absent in Teleosts. Ohta[51] (1939) reported cholic acid as well as scymnol in the bile of the Japanese shark, so apparently elasmobranchs may be capable of forming true bile acids. Scymnol occurs as the sulphuric acid ester, which is more soluble than the free substance. Similar esterification, giving increased solubility is seen in the related compounds, the bile acids, which are conjugated with the amino acids, glycine and taurine. Kazumo,[52] (1940) regarded the scymnols as partial oxidation products in the transition between sterols and bile acids, and described other possible oxidations by which C_{24} bile acids could be produced from substances like 7-dehydrocholesterol. However, there is no very convincing evidence for this theory and it is more likely, on general grounds that bile acids and cholesterol have a common precursor.

In Teleosts. Table 5, 1(B)

Cholic and deoxycholic acid have been found in the bile of the bony fish, and the parallelism between the possession of a bony skeleton and the secretion of bile acids is interesting.

Cholic and deoxycholic acid have been isolated from the biles of the Teleosts investigated. (Table V) A possible intermediate between 7-dehydrocholesterol and isocholic acid was isolated from the bile of the 'Gigi-fish' (Ohta,[75] 1939). A tentative structure for this substance was tetrahydroxynorsterocholanic acid with hydroxyl groups at positions 3,6,12 and 24. Hydroxyl groups at position 6 are only found in the bile acids of the hog. IsaKa & Azato[76] (1940) found this substance in the biles of the 'Bari' & 'Fugu' fish, as well.

Tetrahydroxynorsterocholanic acid.

Bile acids in this group are without exception, conjugated with taurine, even in the case of Plecoglossus, the "sweet fish", which has an entirely vegetarian diet. (Kobayashi,[59] 1927)

c) In Amphibians. Table 6. 11(a)

Tetrahydroxybufostane, $C_{27}H_{48}O_5$; isolated from the winter bile of toads (Makino,[81] 1933) is a compound related to both scymnol and the bile acids. Bufodeoxycholic acid, $C_{24}H_{40}O_4$ (Okamura,[80] 1928,) was oxidised to dehydrobufodeoxycholic acid, an isomer of deoxycholic acid.

TABLE 6.

BILE ACIDS IN 2a) AMPHIBIANS.

Animal	Bile Acids & related compounds	Type of conjugation		Authors	Year	Ref.
FROG Rana catesbiana Shaw	Unspecified Tetra & Trihydroxycholane Trihydroxybisnorsterocholanic acid	T		Moleschott Kazuno & Kuraski Mabuti, H.	1852 1939 1941	77 78 79
TOAD Bufa vulgaris japonica	Bufodesoxycholic $C_{24}H_{40}O_4$		From winter bile	Okamura, T.	1928	80
	Tetrahydroxybufostane $C_{27}H_{48}O_5$		related to bile acids & scymnol	Makino, A.	1933	81
TOAD	Trihydroxyisosterocholenic acid Trihydroxybufosterocholenic acid		Intermediaries between sterols & bile acids.	Shimizu, T. & Kazumo, M.	1936	82

Two structural intermediates, between sterols and bile acids, have been found in toad bile by Shimizu & Kazumo[82] (1936)

Trihydroxybufosterocholenic acid

Trihydroxyisosterocholenic acid

Frog bile yielded a bile acid (Moleschott,[77] 1852) which was not further investigated. Kazuno[78] (1939) isolated the sulphuric acid ester of an unsaturated substance, $C_{24}H_{41}O_3$ from the bile of Rana Catesbiana Shaw. From this he obtained the tetrahydroxycholane.

TABLE 7.

BILE ACIDS IN 2b) REPTILES.

Animal	Bile Acids & Related Compounds	Type of conju-gation.		Authors	Year	Ref.
TURTLE Amyda japonica Emys orbicularis	Tetrahydroxysterocholanic lactone " " and trihydroxy-compound.			Yamasaki, K. Kim, C.	1936 1939	83 84
SNAKE Boa Python (phyton tigris)	Unspecified.	T		Schlieper Schlossberger	1846 1857	85 86
Elaphequadrivigata Elaphecarmata Bothrops alternata. Bungarus mulctitinctus. Crotalus terrificus.	Cholic Cholesterol only Cholic "	T		Iwato & Watanabe Imamura, H. " "	1935 1940	87 88

Mabuti[79] (1941) also isolated trihydroxybisnorsterocholanic acid from the bile of Rana Catesbiana Shaw.

d) In Reptiles. Table 7. (b)

Tetrahydroxysterocholanic lactones have been isolated from the bile of AmydaJaponica (Yamasaki,[83] 1936) and Emysorbicularis (Kim,[84] 1939). Cholic acid is the only bile acid which has so far been isolated from snake bile.

Table 8. BILE ACIDS IN BIRDS

Bird	Bile Acid	Type of conjug- ation.		Author	Year	Ref.
GOOSE	Chenodeoxycholic acid	T	Char avian bile acid	Marrson	1849	89
HEN	Apochenodeoxycholic Gallodeoxycholic Taurocholic Isolithocholic	T	Same as cheno deoxy cholic	Takahashi Yonemura Yamasaki Hoshima T	1938 1928 1933 1930	90 91 92 93
DUCK	Cholic Chenodeoxycholic	T	Always conjug ated with Taurine	Mori T.	1938	94
TURKEY	Cholic Chenodeoxycholic	T		" "	"	
PHEASANT	Cholic Chenodeoxycholic	T		Ohta K.	1939	95

TABLE 9. CONT'D.

BILE ACIDS IN ANIMALS A) HERBIVORES.

Animal	Bile acids & derivatives	Type of conjuga-tion.		Author	Year	Ref
Ox (cont)	3-hydroxy-12-ketocholanic.			Wieland & Kishi	1933	111
	7-12-dihydroxy-3-ketocholanic.		Small amts. about 5 mg./litre.	Haslewood, G. A. D.	1946	112
	3:12-dihydroxy-7-ketocholanic. Sapocholic			Wieland & Hanke.	1936	113
	Sterocholic acid $C_{28}H_{46}O_4$		Related to sterols in composition & bile acids in properties.	Wieland & Kishi.	1933	111

TABLE 9.

BILE ACIDS IN ANIMALS A) HERBIVORES.

Animal	Bile acids & derivatives.	Type of conjuga-tion.		Author	Year	Re
Marsupial Kangaroo	Cholic. Deoxy-cholic. Cheno-deoxycholic.	G. T.		Schlossberger Kimma, T.	1859 1937	10 11
Mammals Hare	Deoxycholic Tetrahydroxy-cholanic.		Possibly a parent sub-stance. Com-plete absence of cholic.	Windaus, A. van Schoor, A.	1928	9
Rabbit	Cholic α-&β lagodeoxy-cholic.		Cholic was ori-ginally thought to be absent.	Kishi S. Ishimo, N.	1936 1938	9 9
Guinea-Pig	Chenodeoxy-cholic 3-hydroxy-7-ketocholanic	T	No cholic acid. Keto-acid an intermediate compound.	Imai, I.	1937	102
Nutria-Rat Myocastor Coypus.	Nutria cholic	G	$C_{24}H_{40}O_5$	Brigl, P. Benedict, O	1933	10
Antelope	Cholic Deoxy-cholic.			Windaus, A. van Schoor.	1928	9
Goat	Cholic Deoxy-	G. & T	Deoxycholic characteristic acid. Stones in 2nd stomach.	Bentsch Schrenk	1848 1925	10 10
Sheep	Cholic	T		Bentsch	1848	10
Musk-Ox	Cholic Deoxy-cholic.	G. & T.		Hammarsten	1904	10
Ox	Cholic Deoxycholic Lithocholic Chenodeoxy-cholic	G. T.		Bentsch Lachinov Wieland & Weyland. Wieland & Revery	1848 1885 1920 1924	10 10 10 11

c) In Birds. (Table 8)

The characteristic avian bile acid is chenodeoxycholic, first isolated from goose bile by Marrson[89] in 1849. Cholic acid occurs with it, in several species. Takahashi[90] (1938) reported a bile acid which he called 'apochenodeoxycholic' from hen bile.

Isolithocholic was found in hen bile in 1930 by Hoshima[93].

Bile acids in birds are conjugated with taurine, not glycine.

f) In Mammals. (Table 9)

The mammalian species investigated have been grouped according to diet, A, Herbivores and B, Carnivores and Omnivores. Glycine conjugation predominates among Herbivores, Table 9 A, but in all carnivorous animals the bile acids are conjugated with taurine. Some omnivores have both types of conjugation.

The Hare and the Rabbit

In hare's bile, Windaus & Van Schoor[96] (1928) found deoxycholic acid and a tetrahydroxycholanic acid, possibly a parent substance. There is no cholic acid, as in guinea pigs, which may be related to the lavish bile flow in these animals. Rabbit bile was also thought to contain no cholic acid, but Ishimo[97] (1938) succeeded in isolating a small quantity. A carbonyl precursor of the bile acids was suspected in rabbit bile, but has not yet been isolated. Deoxycholic acid (Okamura[98], 1930) and Lagodeoxycholic acid are also present. α & β lagodeoxycholic acids, the characteristic bile acid in the animal were isolated by Kishi[99] (1936) and separated by solubility differences. The constitution of these acids

has not yet been elucidated. The α acid (mp 156°) is non-precipitable by digitonin and the β acid melts at 213°. This resembles a substance reported from Munich by Dorrer[100] (1932) from ox bile. Kishi claimed that β-lagodeoxycholic acid was the 12-epimer of deoxycholic acid but Reichstein[101] (1942) & Koechlin compared the melting points and other properties of these compounds and proved that they were not the same substance.

α-lagodeoxycholic

Deoxycholic

β-lagodeoxycholic m.p. 213°

In the Guinea-Pig.

Guinea pig bile contains a hydroxyketoacid, 3-hydroxy-7-ketocholanic acid, isolated by Imai[102] (1937). Chenodeoxycholic acid is also present.

In the Ox

Since large amounts of ox-bile are easily obtainable, the bile acids have been thoroughly investigated. Cholic acid was reported before 1848 and both types of conjugation recognised. Lachinov[108] (1885) isolated deoxycholic acid. In 1911[114] Fischer obtained lithocholic acid from ox gallstones, and in 1920 Wieland and Weyland[109] isolated the same acid from oxbile. Traces of chenodeoxycholic acid were reported by Wieland and Revery[110] (1924) and according to Saba[115] (1939) large amounts may be present. Small quantities of sterocholic acid and 3-hydroxy-12-ketocholanic acid were found by Wieland and Kishi[111] (1933) and "Saphocholic acid" $C_{28}H_{46}O_4$ by Wieland and Hanke[113] (1936). Haslewood[112] (1946) isolated 7:12-dihydroxy-3-keto- and 3:12-dihydroxy-7-keto-cholanic acids, which occur in very small amounts.

Table 10B. Carnivores & Omnivores

In the Hog

The bile acids in the hog are distinctive and possibly related to the metabolic peculiarities of the species. The characteristic bile acid is 3:6-dihydroxycholanic acid, Hyodeoxycholic acid (Grundelach & Strecker,[116] 1847). Fieser[13] reports that the hippopotamus also possesses hyodeoxycholic acid, but does not give a reference. Both α and β forms of the acid were found, the β- acid by Kimura[117] (1937). Chenodeoxycholic acid was isolated from swine gall by Ido and

34

TABLE 10 B.

CARNIVORES AND OMNIVORES.

Weasel	Cholic	T		Sihn, T & Taka, T.	1937	123
Marten	Cholic Deoxycholic			Ohta, K.	1937	53
Otter	" "			Sihn, T.	1937	123
Fox	" "			" Kihara, Y.	1937 1938	123 124
Dog	" "	T		Strecker	1849	125
Seal	x phococholic	T	Hydroxyl groups at 3, 7 & 23 m.p. 222°	Hammarsten Windaus & van Schoor, A.	1909 1928	126 96
Walrus	s "			Windaus & van Schoor, A.	1928 1938	96 127
Leopard		T		Kimura, T.	1937	117
Lion		T		Tanaki, K.	1931	128
Polar Bear	ursodeoxycholic	T	Naturally occurring	Hammarsten Schoda Kazumo, K.	1901 1927 1929	129 130 131
	Cholic, chenodeoxycholic	G	Ursodeoxycholic conj. with glycine.	Kazino Iwesaki Miyazi, S. & Isah	1931 1935 1937 1939	132 133 134 135
Hog	- Hyodeoxycholic	G	Characteristic acid 100% glyco conjugation	Gundelach & Strecker	1847	116
	B-Hyodeoxycholic Lithocholic 3-hydroxy-B-keto-cholanic		Intermediate	Kimura, T. Schoenheimer Fernholz	1937 1937 1935	117 122 119
	Chenodeoxycholic			Ido & Sakurai	1939	118

Ape - pithecus cyclopus	Cholic Deoxycholic Chenodeoxycholic			Mori, T.	1938	137
Capuchin Monkey - Cebus fatuel-lis.	Cholic Deoxycholic			Beck, F.	1940	136
Man	Cholic Deoxycholic Chenodeoxycholic Lithocholic		Only a trace.	Before 1847 Cohn, L. Weiland & Revery Roger	 1894 1924 1928	 138 110 139

Sakurai,[118] (1939).

(α) (β)

α & β Hyodeoxycholic acid

The corresponding 3-hydroxy-6-ketoacid was isolated by Fernholz,[119] (1935) and also by the p-carboxyphenylhydrazine method, Anchel and Schoenheimer,[120] (1938). The configuration of this acid was studied by Sugiyama,[121] (1937). Lithocholic acid was obtained from hog gallstones by Schoenheimer & Johnston,[122] (1937). The incidence was small, 7 stones in 6,150 hogs, but the authors suggest lithocholic acid is also present in normal hog bile.

3-Hydroxy-6-ketocholanic acid.

The other peculiarity is that hog bile is devoid of taurine which would make metabolic investigations interesting, since

such a clear cut differentiation in conjugation is rare. However, in a more recent analysis, Irvin[140] (1939) reports that 94% of the bile acids were combined with glycine, and 6% with taurine.

Dried hog bile had the same choleretic effect on the bile output of fistula dogs, as dried ox bile. (Bermann Snapp and Ivy,[141] 1941). Pure hyodeoxycholic had the same choleretic effect as cholic acid, but this was unaffected by oxidation. This is in contrast to the results obtained with ox bile. (Bermann, Snapp & Ivy,[142] (1940).

The Bear. The characteristic bile acid of bear's bile is ursodeoxycholic (Hammarsten,[129] 1901 & Schoda,[130] 1927), a naturally occurring epimer of chenodeoxycholic acid. This was confirmed by Iwasaki,[133] (1935).

CH_3 R CH_3 H OH OH H

3 -α-β-7-dihydroxycholanic
(ursodeoxycholic)

CH_3 R CH_3 H HO H OH

Chenodeoxycholic acid.

Glyco-ursodeoxycholic acid was reported by Miyazi,[134] (1937) Chenodeoxycholic was also found. Shoda[130] (1927) gave Taurine as the predominant amino acid in bear's bile.

I.4. THE PHYSIOLOGICAL IMPORTANCE OF "BILE SALTS"

The great surface activity of bile salts is largely responsible for their physiological importance. Accounts of the properties of bile salts can be found in standard works such as Sobotka[143] or Best and Taylor[144]. Their importance in jaundice is reviewed by Ottenberg[145] (1938).

Although earlier workers had thought bile to be essential for life, Scott,[146] (1945) succeeded in maintaining bile fistula dogs in perfect health, provided they were given adequate amounts of the fat soluble vitamins, intravenously. In these dogs, the essential part played by the bile was simply to facilitate the absorption of Vitamins A, D, K and E. Steroid hormones can, however, be absorbed in the absence of bile. (Seyle,[147] (1943). Von Fuerth,[148] & Minnibecke, (1930) and Verzar,[149] (1931) rather over-emphasised the importance of bile in fat absorption. Josephson and Rydin,[150] (1936) showed a difference in the bile acid levels in the portal and systemic blood streams, the portal values being considerably higher. This agreed with Riegel's,[151] (1935) findings and was later confirmed by Jenke and Graff,[152] (1939). The hypothesis that a bile acid/fatty acid complex passed through the intestinal villi, after which separation occurred, was not confirmed by Frazer,[153] (1943 [illegible]). He found that neutral fats and fatty acids were not absorbed in the same way.

Lipolysis being a determining factor in the path of absorption[154] (1943 [illegible]). Emulsification, however, was greatly influenced by bile salts and the optimum conditions were given by bile salts/fatty acid/monoglyceride, over a pH range 4-8.5, a system which is independent of any pH change which might occur in the intestine (Frazer,[155] 1944).

I.5. ACTION OF BACTERIA ON BILE ACIDS

1. Reduction. Chenodeoxycholic acid was obtained by the bacterial reduction of dehydrochenodeoxycholic acid, using B. coli communis. (Sihn,[156] (1938). The bacterial reduction of dehydrocholic acid was investigated by Fukui,[157] (1937), working with B. coli communis from faeces, & Mori,[158] (1939) with Proteus vulgaris. In both cases the same reduction product 7:hydroxy 3:12:diketocholanic acid, was obtained.

Dehydrochenodeoxycholic

Chenodeoxycholic

The selective reduction of the Keto group at C_7 also takes place in the animal body (Fukui & Ishada,[159] 1937). The results should be compared with those of Yamasaki & Kyogoku[160] (1937).

Dehydrocholic acid

7-hydroxy-3:12-diketo-cholanic acid.

2. Oxidation In 1925 Koozoo Kashiro[161] investigated the action of B. coli communis on cholic acid, apparently hoping to obtain the same oxidation products that Tauzer[162] (1909) and Wieland & Schultz,[163] (1923) isolated after chemical oxidation with nitric acid. All he reports is the isolation of a crystalline acidic substance m.p. 198°. although Rosenthal, Wislicki & Pommerelle,[169] (1927) & Bollmann, & Mann,[164] (1933) thought that the liver played a predominant part in the destruction of bile salts, Schmidt and Hughes,[166] (1942) showed that cholic acid incubated with liver in vitro is in no way destroyed. In guinea pigs

the caecum plays an important part, since if it was rendered functionally inactive by ligation, 88% of the administered bile acid could be recovered, whereas in the intact animal recovery was only 45%. Cholic acid was then shown to be destroyed in the isolated caecum, and in media incubated with a suspension of caecal contents. The heat labile organism responsible was Alcaligenes faecalis. Schmidt and Hughes[167] (1942) then investigated the factors influencing the catabolism of cholic acid by pure strains of Alcaligenes faecalis. They found that the oxygen supply, the concentration of cholic acid and the strength of the innoculum were all of importance. A. faecalis cultured in serum was also found to oxidise deoxycholic acid, hyodeoxycholic acid, lithocholic acid and dehydroisoandrosterone to keto-derivatives, while oestriol and oestradiol were unaffected. The substance giving a negative Gregory and Pascoe reaction which was the end product of this catabolism was found to be 3:7:12-triketocholanic acid. (Hoehn, Schmidt and Hughes,[168] (1944)) The course of the oxidation was then investigated and a stepwise oxidation of the hydroxyl groups postulated, and later proved. The first oxidation product was 3:12-dihydroxy-7-ketocholanic acid, which was not isolated as such, but derivatives such as the monosemi carbazone were obtained. Secondly 3-hydroxy-7:12-diketocholanic acid was

isolated as the disemicarbazone from the reaction mixture. Finally 3:7:12-triketocholanic acid was isolated pure and in high yield.

OH CH3 R CH3 HO O

Cholic Acid.

O CH3 R CH3 HO O

3:12-dihydroxy-7-keto

O CH3 R CH3 O O

3-hydroxy-7:12-diketo

3:7:12 triketo

OXIDATION OF CHOLIC ACID

The hydroxyl groups are therefore attacked in the order 7, 12,3, as in oxidation by chromic acid. It is interesting to compare this with the order of reduction, which occurs first at C_7 in rabbits and by certain bacteria, but at C_3 in toads, by yeast and chemically.

I.

ANIMAL EXPERIMENTS WITH BILE ACIDS AND DERIVATIVES

6.

Shibuya,[169] (1933) injected dehydrocholic acid subcutaneously in toads, and recovered a new acid from the urine. This acid was thought to be 3-hydroxy-7:12-diketocholanic acid, but only a very small amount was isolated. This experiment was repeated by Yamasaki and Kyogoku,[170] (1935) and the identity of the hydroxy acid confirmed. Dehydrodeoxycholic acid was then given to toads, and 3 (β) Hydroxy-12:ketocholanic acid isolated from the urine. (Yamasaki,[171] 1935b). Since the hydroxy acid could be isolated from the bile, the reduction of dehydroacids was thought to occur in the liver.

Dehydrocholic

3-hydroxy.7:12. diketocholanic

Dehydrodeoxycholic

3-(β) Hydroxy.12.ketocholanic.

Kyogoku,[160] (1937) isolated a mixture of the 3 (α) hydroxy 12 ketocholanic and the corresponding (β) acid, after injection of dehydrodeoxycholic acid. The same worker[172] (1937 b) then gave 3 (α) hydroxy - 7:12 - diketocholanic to toads, but only a very small quantity of the corresponding (β) acid was excreted. He suggested that the greater part of the (α) acid was metabolised by the animal.

α-Form β-Form

Sihn,[173] (1938) injected 3-keto-7:12 dihydroxycholanic acid into toads and reported the excretion of a small amount of free cholic acid, in addition to a considerable quantity of the unchanged keto-acid. Reduction of the Keto group at C_3 had, therefore, occurred.

3-keto-7:12-dihydroxycholanic

Tukamoto,[174] (1939) used 3:6-diketoallocholanic acid, also in toads, to see if a change over from allocholanic to cholanic would occur. Unchanged diketo-acid was isolated

from the bile and there was a very small excretion of the 3 (β) hydroxy-6-keto<u>allo</u>cholanic acid in the urine. Injecting the (β) hydroxy acid was then tried, but it was excreted unchanged. It must be pointed out that this substance is foreign to the organism, and had a marked diuretic and choleretic effect on the experimental animals. Mabuti[175] (1941) gave 3α-6-α dihydroxycholanic acid to frogs (Rana Catesbiana Shaw) Inversion occurred at C_3, but not at C_6.

3:6:Diketo<u>allo</u>cholanic

3:Hydroxy-6-keto<u>allo</u>-cholanic

Apocholic acid could be changed to dihydroxycholenic acid by the action of ultra violet light or sunlight (Sihn,[176] (1939) so it was of interest to see if this change occurred in the animal body. Apocholic was given to toads (Sihn,[177] 1939b) and small amounts of the dihydroxycholenic acid were isolated from the bile. Toads were also used in an experiment with 'sitosterol' by Ashikari,[178] (1936) who found that the sitosterin

Apocholic acid

passed through the liver and was excreted unchanged in the bile. No intermediaries could be isolated from the urine or faeces.

Dihydroxycholenic acid

A similar series of experiments was carried out with rabbits. Fukui & Ishada,[179] (1937) injected rabbits with dehydrocholic acid. Some was excreted unchanged, but a small amount of 7-(β) hydroxy-3:12-diketocholanic acid was isolated from the urine. Selective reduction at C_7 had occurred in the mammalian organism, whereas in the toads, C_3 was attacked first. Kim,[180] (1938) reported the isolation of deoxycholic acid from rabbit urine after giving dehydrocholic acid.

7-(β)hydroxy-3:12-diketocholanic acid.

The author mentioned that glycodeoxycholic acid is present in rabbit bile, and it would be possible for deoxycholic acid to be split off from this by 'glycocholase' in the kidney. The same

Dehydrocholic

Deoxycholic

result was obtained when dehydrodeoxycholic acid was injected. Miyazi[181] (1938) isolated 3(β)-6(α)dihydroxyallo-cholanic acid from rabbit urine, after giving 3:6-diketo-allocholanic acid. Epimerisation at C_3 had occurred, but there was no change over from allo-cholanic to cholanic.

3:6-diketoallocholanic acid   3(β)6(α)Allodihydroxycholanic

Since the change over from a sterol such as ergosterol in the food, to a bile acid, hydrogenation of a double bond must occur, an unsaturated ketoacid 12-ketocholenic acid was given to the animals (Mori,[182] (1939). A very large yield of deoxycholic acid was obtained, so that probably some of this was from existing glycodeoxycholic acid.

12-ketocholenic acid.

Kim[183] (1939) used guinea pigs in a metabolic experiment. Cholic acid, which is not present in guinea pig bile was injected, to obtain information about the formation of

deoxycholic from cholic. He found scarcely any deoxycholic in bile or urine, but based a hypothetical reduction theory on the occurrence of derivatives similar to the 3-hydroxy-7-diketocholanic acid isolated by Imai[102] (1937). Partial oxidation of cholic acid is postulated, to give 3-hydroxy-7:12-diketocholanic. Complete reduction at C_7, giving 3-hydroxy 12 ketocholanic, and then partial reduction of the Keto group at C_{12}.

Cholic Acid

partial oxidation

3-hydroxy-7:12-diketocholanic

3-hydroxy-12 ketocholanic

Deoxycholic acid

Similarly Chenodeoxycholic could be formed from cholic acid, and in this case the intermediate acid is the 3-hydroxy-7-ketocholanic mentioned above.

Cholic Acid

3-hydroxy-7:12-diketocholanic

3-hydroxy-7-ketocholanic

Chenodeoxycholic

It appeared from these experiments, that reduction of keto-acids did not occur in the same way in mammals as in amphibians. Inversions at C_3 occurred in both groups of animals, but in neither was there any change from the allocholanic to cholanic series.

I.

7) THE EFFECT OF BILE SALTS a) Orally

a) This was investigated in dogs by Doubilet[184] (1937[185]). When deoxycholic acid and cholic acid were given to a bile fistula case the out-put of both acids in the bile was increased. When a large quantity of deoxycholic acid was given, although the total output of bile acids increased, the output of cholic acid fell. A proportionate increase in conjugated bile acids was observed, but this was entirely as glycocholic acid. This increase in conjugated acids did not occur when cholic acid was fed. In bile fistula dogs, various pure bile acids were given orally, and the biles analysed by Doubilet's[186] (1936) method. The observations on man, quoted above, were confirmed and the output of cholic and deoxycholic acid found to be complementary. The total output of bile acids remained constant. Natural dog-bile salts produced the largest output of concentrated bile, unconjugated bile acids had less effect, while keto-acids had the least action. However, Bermann, Snapp and Ivy[142] (1940) reported different results using the same experimental animal. They found that unconjugated mono-, di-, or tri-keto-acids had the greatest choleretic effect, while conjugated bile salts and 'oxidised bile salts' had a similar, less marked action. Possibly choleretic activity was slightly suppressed by conjugation. Apparently, the biliary cholic acid increase

came only from the cholates fed, synthesis by the liver being unaffected. These workers suggested that the liver dealt differently with keto-bile acid as opposed to hydroxyacids. The small increases in the amount of keto-bile acids after cholates were fed was probably due to oxidation in the liver.

b) Intravenously in animals and man The liver was thought to dispose of peripherally injected bile salts. Chabrol, Cottet and Sallett[187, 188] (1936). If a considerable quantity of sodium cholate was injected in rabbits, the greater part disappeared from the blood stream in 4 minutes and after 30 minutes, the blood cholates had returned to the pre-injection level. Josephson, Jungner and Rydin[22] (1938). When the liver vessels were ligated, the blood levels were still much lower than one would expect if the cholate were simply diluted by the blood. Fixation of the cholates by the tissues was suggested as an explanation. Josephson[23] (1938) working with cats with experimental obstructive jaundice, found no such disappearance of the injected bile acids, and the concentrations did correspond with simple dilution figures. He concluded that in normal animals a fixation mechanism was present which was absent in jaundice. Absorption on vessel walls which occurred in normals, was prevented in jaundice as the tissues were already saturated with bile salts.

Josephson, Jungner & Rydin[22] (1938) found that if cholates were injected directly into the portal vein in normal rabbits, the expected decrease did not occur and blood levels remained high for some time. This did not occur in cats. They suggested that local flooding of the liver with a high concentration of bile salts paralysed the excretory mechanism. The quantitative excretion of administered cholates by the liver, was shown by Bollman and Mann[189] (1936) & Josephson & Rydin[22] (1938). This was confirmed under more physiological conditions by Josephson & Larsson[23] (1939). The effect of dehydrocholates intravenously was investigated by Sterner, Bartle & Lyon[190] (1931). Lichtman[191] (1936) compared the blood clearance and renal excretion of cholic and deoxycholic acid. The blood deoxycholate remained elevated for a considerable time.

c) <u>In Liver Disease</u>. In obstructive jaundice, when the liver parenchyma is uninjured, production and transport of the bile acids still continued. (Whipple and Smith[192] (1930) and Bollman and Mann[193] (1935)) The concentration of cholates in the blood in cases of obstructive jaundice was reported to have reached values as high as 30 mg./100 ml. whereas in hepatitis, 10 mg./100 l was the maximum amount. Josephson & Kaunitz[194] (1937) showed that the hepato-bilio-lymphatic circulation was responsible for this. In cholangitis and

cholecystitis, Doubilet[186] (1936) reported the resorption of 9/10 cholates from the gall bladder and ducts, into the liver. This did not seem to occur in other conditions. The elimination of injected cholates in normal and experimentally jaundiced animals, was compared by Josephson, Jungner and Rydin (1938 [22, 195]). The jaundice was obstructive - ligation of the bile ducts, or from liver poisons such as CCl_4. Different elimination curves in these types of liver disease were reported. The administration of dehydrocholic acid was used as a liver function test by Adlesberg[197] (1924). He reported an increased lowering of surface tension in normals and not in liver disease, which is contrary, both to expectations and to later findings.

In 1934, Nakagawa[198] gave dehydrocholic acid orally and intravenously as a liver function test, and found bile acids present in the urine in cases of liver disease, but not in normals. However, the method of estimation is open to criticism. The elimination in man, after 0.5 g. sodium cholate intravenously was studied by Josephson & Larrson[23] (1939). In 1943, Josephson[196] published results with this test on patients with various types of liver disease, and claimed that the curves obtained were sufficiently different to be of diagnostic use.

I.

8. A BRIEF SURVEY OF THE ANALYTICAL METHODS USED IN THE ESTIMATION OF BILE ACIDS AND BILE SALTS

A. Methods based on the Physical Properties of Bile Salts.

1) Optical Rotation Hoppe-Seyle[199] (1863) related the specific rotation of the bile acids to their concentration in body fluids. This method was modified by Rosenthal[200] (1930) but gave unreliable results.

2) Surface activity The alteration in the surface tension of a liquid by small amounts of bile has been used for many years. Hay's qualitative test for bile salts in urine, depends on this, but even under carefully controlled conditions, i.e. constant pH etc. the results are in no way quantitative. Norgaard[201] (1936) claimed that a quantitative interpretation from Hay's test was possible, and threw some light on liver function. Josephson (unpublished) used a surface tension method for icteric sera which gave results of clinical importance, but he emphasised the relative nature of these values. Kaunitz, Gonzato[202] et al also use a surface tension method for serum. Donnan[203] (1909) investigated the surface tension of urine, using a single drop method, and found wide variations among normals and many interfering factors. Various other workers (Table 17) used more or less the same method and Duco and Panza[204] (1930)

stabilised the conditions by working at pH 2, when the electrolytes in urine no longer interfered. Their readings before and after treating the urine with charcoal, represented the bile acid content. Morrison and Swalm[205] (1939) simplified this method, but they obtain very high normal values, and figures of about 2 gms/100 ml. in urine from jaundice cases. Dognon & Gougerot[206] (1942) use a physical method for estimating qualitatively the bile in urine, by the dispersal of a surface film of oil on water by one drop of the test urine.

3) Haemolysis Lichtman[207] (1934) estimated bile salts by their power to haemolyse blood. He used a stock solution of sheep erythrocytes, expressing the results in terms of deoxycholate. Values for other bile acids could be obtained from 'haemolysis equivalents'.

B. Chemical Methods

1) Foster & Hooper[208] (1919) calculated the concentration of the conjugated bile acids in bile, from the gasometric estimation of the amino acids (Van Slykes[209] method). This, together with a sulphur determination was used by Schmidt & Dart[210] (1921) and Rosenthal[29] (1923). However, the possibility of significant amounts of free bile acids in pathological biles was not taken into account by such analyses.

2) Gravimetric Breusch[211] (1934) used a gravimetric method,

TABLE 11.

COLORIMETRIC ANALYSIS OF BILE ACIDS

Authors	Date	Ref.	Reagents	Colour	Comment
Pettenkoffer	1844	216	Conc. H_2SO_4 Sugar, starch or saccharose.	Purple	Very unspecific
Enderlin	1850	217	Less strong H_2SO_4 or HPO_4	Blue	More specific
Kuelz	1875	218	Fructose		
Drechsel	1883	219	Conc. H PO_4 Sugar	Red	not very sensitive.
Udransky	1888	220	Furfural		Furane ring in the sugar molecule reacted so the aldehyde was used.
Neuberg & Jolles.	1908	221	Conc. H_2SO_4 Furfural	Purple	
Ville & Derrier.	1908-9	222	H_3PO_4 Vanillin anisaldehyde.		
Inouye & Ito	1908	223	Vanillin	Pink	
Herzfeld & Szilard	1924	224	Modified PTK-Enderlin reaction.	Blue	More specific
Szilard	1925	213	Glacial acetic benzoyl peroxide & H_2SO_4	Dark green	
Perlzweig & Barron	1926	225	Acetic anhydride & H_2SO_4	Brown	
Chiray & Cuny	1928	226	H_3PO_4 Furfural	Blue-green	
Gregory & Pascoe.	1929	227	50% H_2SO_4 Furfural	Blue	Specific and sensitive
Nakagawa	1930	228	H_3PO_4 Furfural	Red	
Tashiro	1931	229	H_2SO_4 Furfural	Fluorescence	Unspecific unreliable
Reinhold & Wilson	1932	230	H_2SO_4 Acetic Acid Furfural	Blue	Colour better in presence of acetic acid.
Yamasaki	1933	231	Hc. modified Hammarsten	Blue	
Kusui	1933	232	Hc. Cane sugar	Orange	
Scott	1934	233	HCl. or H_3PO_4 Fructose	Red-purple	Unspecific
Chabrol et al	1934	234	H_3PO_4 Vanillin	Pink	Not very sensitive.

but high vacuum separation of the fatty acids was first necessary. Vitali[212] (1881) suggested the use of the highly insoluble alkaloid salts of the bile acids, but this has not apparently been followed up.

3. Volumetric Szilard[213] (1926) converted the bile acids into their ferric salts and analysed them by an iron titration method. Scrupulous analytical precautions were necessary, and blood levels by this method were high. Katayama[214] (1928). Peoples[215] (1927) incorporated thioglycollic acid into the procedure, the iron compounds being insoluble in water but soluble in 95% alcohol.

4. Colorimetric Methods in which the bile acids were determined colorimetrically proved the most promising. The original (1884) Pettenkoffer[216] reaction is the basis of most modern methods. Table 11 gives the references to the reagents employed in these colour reactions.

The greater specificity in the more recent analyses was obtained by careful adjustment of the reagent concentrations and by elimination of interfering substances. Schmidt[235 & 236] (1937) and (1942), Josephson[237] (1935), Irwin Johnston & Kopala[238] (1944). Hammarsten's[239] (1909) reaction has been modified by Yamasaki[231] (1935). In this case the bile acid is warmed with concentrated hydrochloric acid, and a blue colour developed.

TABLE 12.

STEROIDS GIVING A POSITIVE PETTENKOFFER (SCHMIDT) REACTION.

Apocholic acid
Chenodeoxycholic acid
Cholic acid
nor-cholic
bisnorcholic
3-succinic ester of methyl cholate
3-hydroxy-5-cholenic acid
12:hydroxy-3-cholenic acid
Me12:hydroxy-3-cholenic acid
Triformylcholic
3:7:12-Trihydroxynorcholanyldiphenylcarbinol
Dehydrotransandrosteroneacetate

The orange colour produced by bile acids in the presence of hydrochloric acid and laevulose was used in the estimation of bile salts in blood, by Kusui[232] (1933), but the values so obtained were grossly unphysiological. The colour reactions related to the structure of bile acids and their derivatives:- Kerr & Hoehn[240] (1944), who discussed the specificity of Schmidt's[235] (1937) modification of the Pettenkoffer reaction. The steroid to be tested was dissolved in glacial acetic acid and when the colour had been developed with furfural and sulphuric acid, more acetic was added. They obtained positive results with the steroids listed in Table 12.

They concluded that saturated steroids with a hydroxyl at C7 would give a positive reaction, and also compounds having a double bond in one of its four rings, would therefore be expected to have a positive reaction.

Hammarsten's reaction was positive for cholic acid and its conjugates and esters, scymol, apocholic acid, ethyl cholate, and phocacholic acid. Saba[241] (1940) investigated the reactions of various bile acids and derivatives and compared Hammarsten's test, the hydrochloric acid-laevulose test, and the benzaldehyde reaction with the m-initrobenzene test (Table 13).

Saba attempted to relate the structure of these compounds with their behaviour in these four colour reactions. A positive Hammarsten was only given by bile-acids with hydroxyl groups at C_3, C_7, C_{12}. The hydrochloric acid laevulose test was only posi-

TABLE 13.

COLOUR REACTIONS OF VARIOUS BILE ACIDS.

Compound	Hammarsten	HCl-Laevulose	Benzaldehyde		M-Dinitro Benzene
			Immediate	Delayed	
Cholic & Conjugates	Positive	Positive	-	-	-
Deoxycholic	-	-	Positive	-	-
Chenodeoxycholic	-	-	Positive	-	-
Ursodeoxycholic	-	-	-	Positive	-
Hyodeoxycholic	-	-	-	Positive	-
Dehydrocholic	-	-	-	-	Positive
Dehydrodeoxycholic	-	-	-	-	Positive
Dehydrohyocholic	-	-	-	-	Positive
Dehydrochenocholic	-	-	-	-	Positive
β-Lagodeoxycholic	-	-	Positive	-	-
3:Keto-12:Hydroxycholanic	-	Positive	-	-	Positive
3:Keto-7:12-DI-Hydrocholanic	-	-	Positive	-	Positive

tive in the presence of hydroxyl groups at C_7 and C_{12}. Substances giving the "Immediate benzaldehyde reaction" must have a hydroxyl at C_{12}, a hydroxyl or keto group at C_3 and no group at C_7. As choleic acid gave a positive reaction, fatty acids do not interfere. The "Delayed benzaldehyde reaction" was positive for bile acids with no group at C_{12} and hydroxyls at C_3, C_6 and C_7. There was an unexpected and inexplicable exception in the case of -3-6-dihydroxy cholanic acid, which gave a negative reaction, unlike the -acid. Woker & Antener[242] (1939) reviewed the colour reactions of the sterols. Miescher[243] (1946) tabulates some of the colour reactions of bile acid derivatives.

The colours given by some of the commoner bile acids with these reactions are shown in Table 14.

TABLE 14.

COLORIMETRIC ANALYSIS OF BILE ACIDS.

Bile Acid	Colour Reaction	
	Reagents	Colour
Cholic and Conjugates	Pettenkoffer Sugar & H_2SO_4	Purple
	Gregory & Pascoe Furfural 50% H_2SO_4	Blue
	H_3PO_4 & Vanillin	Pink
	H_3PO_4 & furfural	Red
	H_3PO_4 furfural	Blue-green
	Hammarsten HCl	Blue
	HCl - laevulose	Orange
	H_3PO_4 - laevulose	Red-purple
Deoxycholic	H_3PO_4 & vanillin	Pink
	Benzaldehyde & H_2SO_4	Green
Chenodeoxycholic	Benzaldehyde H_2SO_4	Violet
	H_2SO_4 p-dimethylbenzaldehyde	Purple
Hyodeoxycholic	Benzaldehyde H_2SO_4	Violet
Apocholic	PTK Hammarsten	
Keto-acids	m-dinitrobenzene	Orange

I. 9 a)

Blood Levels of Bile Salts in a) Normal Blood.

Various unspecific methods were used, and interference from cholesterol, lipoids, etc. made the results unreliable. The levels found, together with the methods used, are given in Table 15. Many workers failed to find any trace of bile acids in normal blood, including Walker [244] (1930) who attributed some of the positive findings with the original Pettenkoffer reaction, to Cholesteryl and Barium oleates.

TABLE 15.

BILE SALTS IN NORMAL BLOOD.

Author	Date	Method	Level in mg./100 ml.	Ref.
Chabrol & Benard	1921	Spectroscopic method.	0 - 2.0	245
Greene, Aldrich & Rowntree	1927	Modified Pettenkoffer reaction.	2.5 - 6.0	246
Charlet	1929	Pettenkoffer	3.0 - 4.5	247
Coquelet	1927	Modified Gregory & Pascoe. ? (1929)	2.8 - 4.0	248
Schalscha & Lande.	1927	Foster-Hooper Gasometric method.	2.6 - 5.6 5.0 - 12.0	249 214
Katayama	1928		5.0 - 12.0	214
Tashiro	1931	Fluorescence	60 - 100.0	229
Scheinfinkel	1933	Spectroscopic	0.5 - 0.8	250
Kusui	1933	HCl and Laevulose	7.6 - 11.0	232
Lichtman	1934	Haemolysis	0.5 - 4.0	207
Chabrol & Charonnet	1934	Phosphoric acid and vanillin.	0.4	234
Josephson	1935	Modified Gregory & Pascoe.	0.6 - 2.5	237
Ohyama	1938	Laevulose - HCl		251
Gigon & Noverraz	1940	Surface tension method	0 - 1.0	252

TABLE 16.

BILE ACIDS IN ICTERIC BLOOD.

Authors.	Year	Method	Bile acids mg/100 ml.	Liver Disease	Ref.
Gilbert, Chabrol Bénard.	1920	Pettenkoffer.	10		253
Herzfeld & Haemmerli.	1925	Modified Pettenkoffer.	3-12.5	Icterus neonatorum.	224
Rosenthal & Wislicki.	1926	Gasometric.	av. 6.5		255
Schalscha & Landé.	1927	Gasometric.	" 15	Obstruction.	249
Rosenthal & Wislicki.	1927	Gasometric.	" 10	Hepatitis.	254
Rowntree, Greene & Aldrich	1927	Modified Pettenkoffer.	4-8	Cirrhosis with ascites.	246
			5.5-16.3	Obstructive jaundice.	
			3.5-15.3	Non-obstructive jaundice.	
			5.1	Haemolytic jaundice.	
Chabrol et al.	1934	Phosphovanillin	24	Weil's.	234
			2.5-50	Frank icterus.	
			up to 9	Cirrhosis.	
Katayama.	1928	Method not published.	18-48	Acute obstruction.	214
			14-30	Chronic obstruction.	
			22-33	Cardiac disease.	
Lichtman.	1934	Haemolysis.	up to 10	Liver disease.	207
Josephson.	1939	Modified Gregory & Pascoe.	1.9-10.4	Obstruction.	255
			0.3-8.9	Hepatitis.	
			2.6-6.7	Cirrhosis.	
			1.7-4.9	Sec. liver symptoms.	
Gigon & Noverraz.	1940	S.T. lowering of serum.	0.1-22.0	Liver disease.	252

b) Icteric blood - Table XVI.

c) In urine - Table XVII

The estimation of bile salts in the presence of urinary pigments presents a very difficult problem. Removal of the pigments have been accepted in the application of the various colour reactions to urines. Dragendorf [256] (1869) used acidification and benzene extraction followed by solution of the bile salts in amyl alcohol, and the original Pettenkoffer on the dried residue. Scott [233] (1934) estimates the bile salts by their colour with hydrochloric acid and laevulose, after a laborious precipitation with ammonium sulphate and soxbilet extraction. Bollman and Mann [189] (1936) applied Gregory and Pascoe's reaction directly to the diluted urine, ignoring the high pigment blanks. Lichtman [257] (1938) mixed the urine with alcohol and employed Schmidt's [235] (1937) modification of Josephson's method, but reports such high values in normal dogs' urine that his results are suspect.

TABLE 17

METHODS OF ESTIMATING BILE SALTS IN URINE

Author	Year	Ref.	Values found: Normal	Values found: Icteric	Method
	1. Surface tension methods				
Shemensky	1920	258			
Doumer & Doumer	1921	259			
Gilbert, Chabiol & Benard	1921	253			
Mueller	1922	260			
Adlesberg	1924	197			
Ducq & Panza	1930	204			
Morrison & Swalm	1939	205	10-350mg./100ml.	Up to 82mg./100ml. 200-400mg./100ml.	
	2. Gasometric				
Schmidt & Merrill	1924	261		Up to 600mg./day	
Rosenthal & Wislicki	1927	254		Up to 250mg./day 25mg./100ml. in obstructive jaundice	
	3. Colorimetric				
Nakagawa	1934	198			Generation of turbidity on adding acetic acid
Scott	1934	233			HCl & Laevulose
Hoppe-Seyle	1860	252		110-340mg./day	Modified Pettenkoffer
Bischoff & Looser	184	264		100-350 " "	" "
Dragendorf	1869	256			Original "
Bollmann & Mann	1936		Undetectable	from 100mg./day (Experimental obstruction)	Gregory & Pascoe
Lichtmann	1938	257	2.5-22mg./100ml. normal Dogs.		Schmidt's modification

2. EXPERIMENTAL.

1. THE ESTIMATION OF CHOLATES IN BLOOD

1). The colour reaction used: Cholic acid and the conjugates glyco- and taurocholates, were estimated by Josephson's (1935) [237] method, with some modifications. In this method, interfering substances are removed from the blood by extraction with ethyl acetate etc. and the blue colour given by bile acids with 50% v/v sulphuric acid and fresh aqueous furfural, is measured in a colorimeter using an appropriate filter.

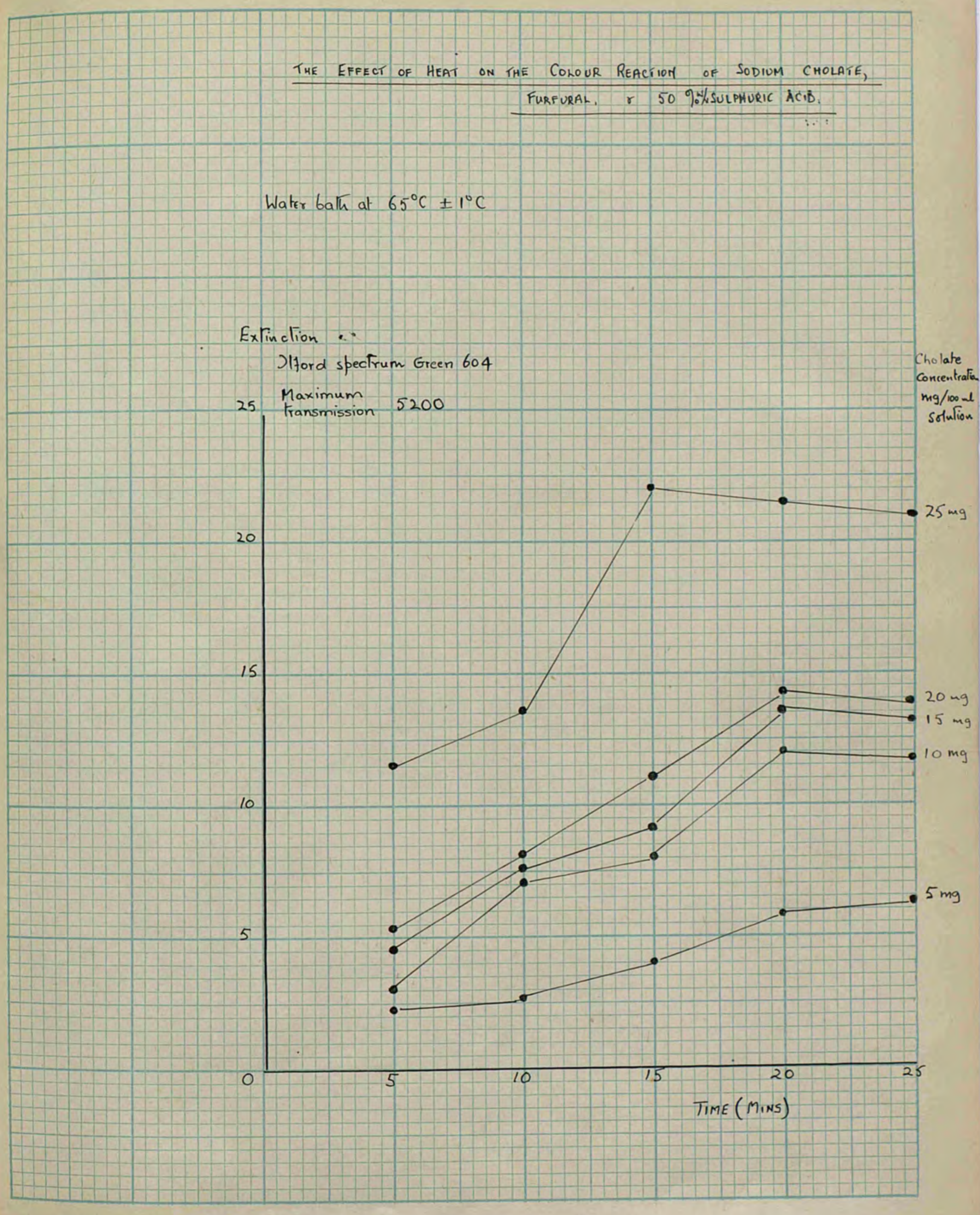
Pure aqueous sodium cholate was used as the standard. 50 mg. cholic acid was dissolved in the theoretical amount of sodium hydroxide, and the volume made to 100 ml. Dilutions of this stock standard were made and 1 ml. portions used for the colour reaction.

The Optimum conditions for the development of the colour

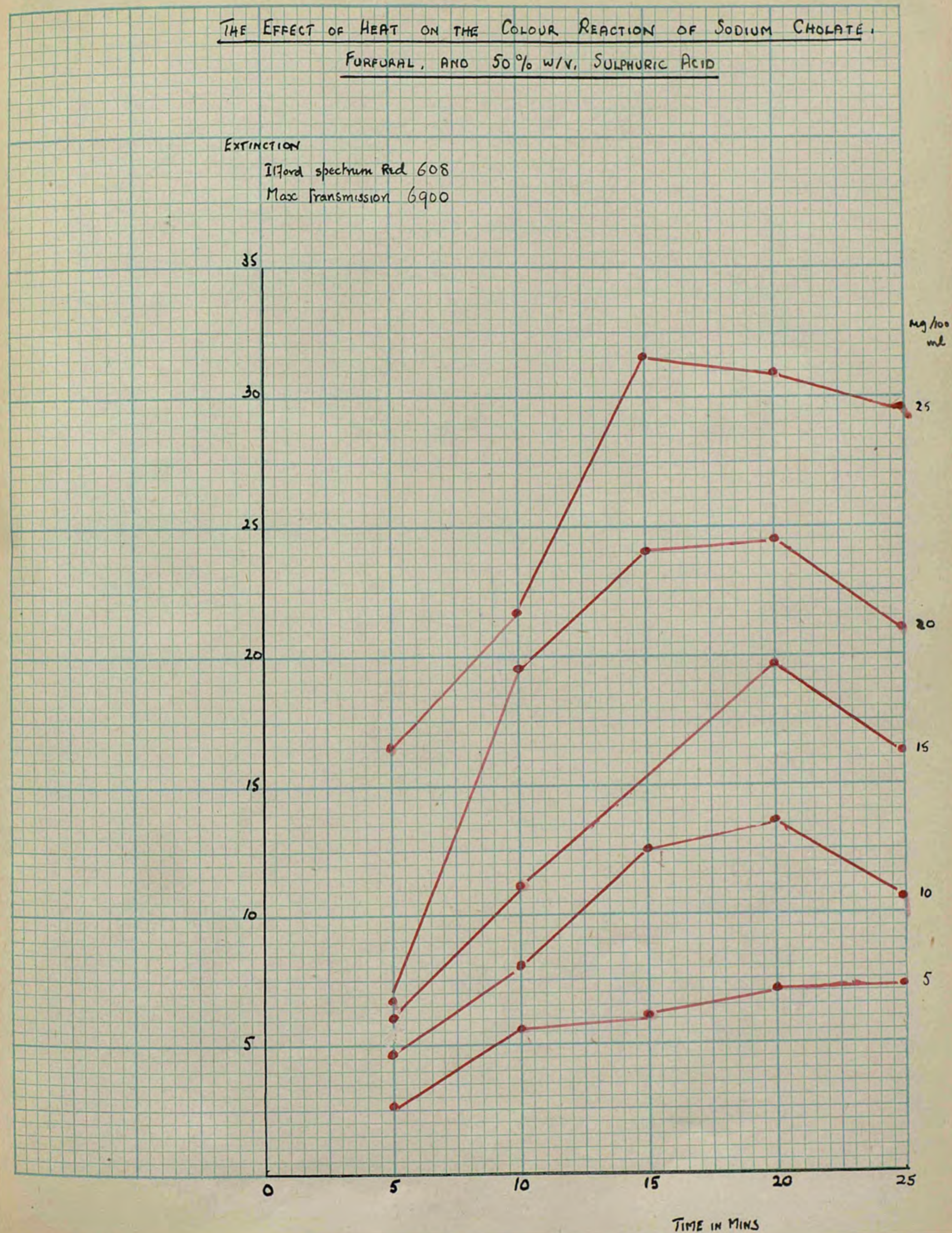
a) Concentration of sulphuric acid. 45% v/v sulphuric acid was recommended by Gregory and Pascoe (1926) [227] for this reaction. The use of too concentrated acids results in charring and brown colours are produced. 55% v/v sulphuric acid gave a less stable blue colour. The most suitable strength of acid was found to be 50%. v/v. 5 ml. of this was added to 1 ml. of standard sodium cholate.

b) Purity of the furfural. Commercial samples of furfural were found to give different colours with the standards. One sample produced purple turbid solutions with pure sodium cholate, instead of the described clear blue colour. The

Graph 1



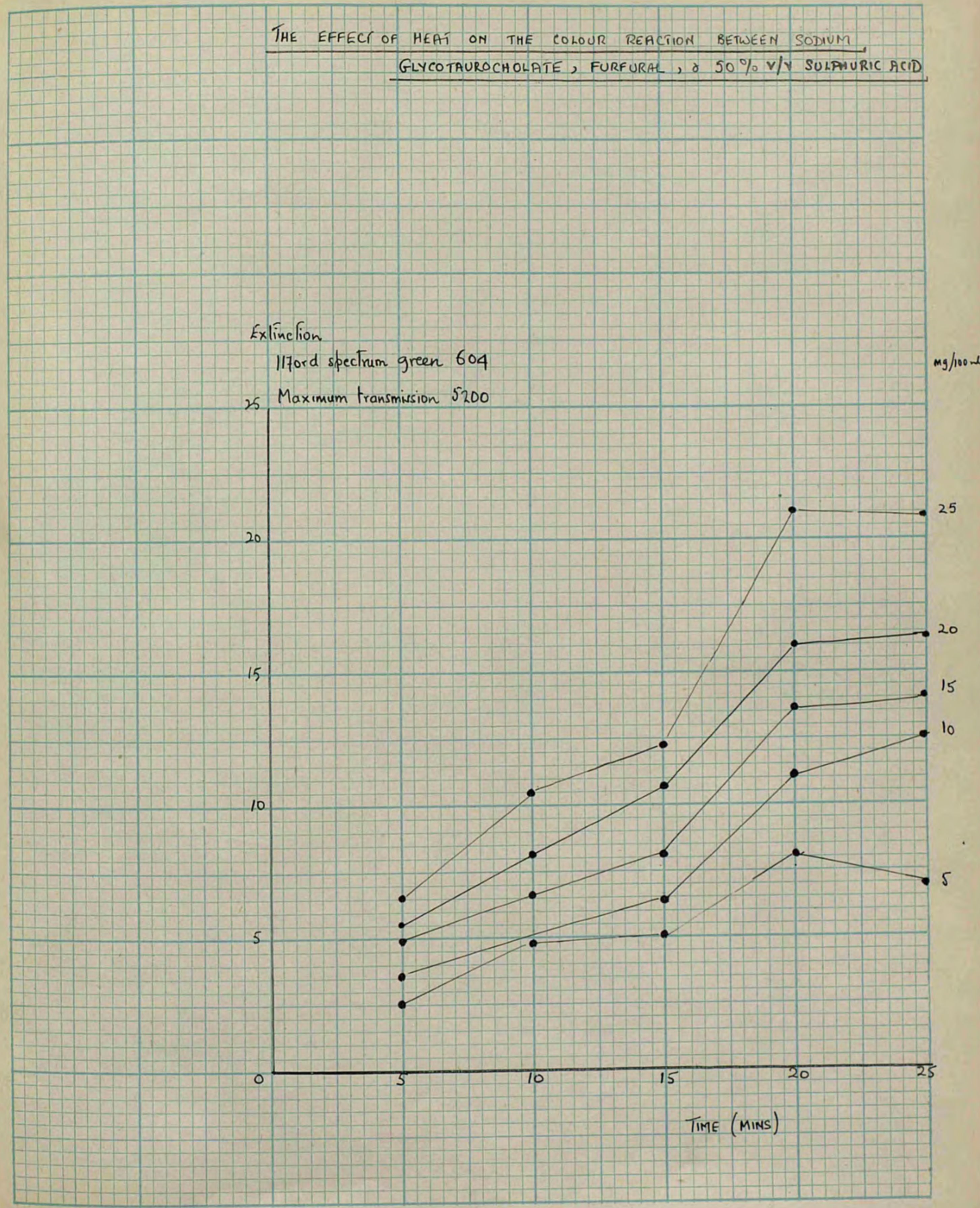
Graph 2



furfural must be redistilled immediately before use and should be a clear, pale yellow, mobile liquid. Redistilled furfural (Hopkins & Williams) was obtained in 100 g. amounts, and 1 ml. redistilled from a 10 ml. capacity Claisen flask in a Rose's metal bath before each test. 0.2 ml. distillate was dissolved in 9.8 ml. distilled water with shaking. 0.5 ml. of this solution (2%) was added to each tube containing cholate and sulphuric acid. (In practice, the acid was pipetted into the standards while the furfural was distilling, as, if the blood filtrates stood too long with the sulphuric acid, brownish colours were sometimes produced.)

c) <u>Duration of heating.</u> The tubes were lightly stoppered with cotton wool, well mixed and immersed to within an inch from the rim, in a metal water bath at $65^{\circ} \pm 1^{\circ}$. Tubes were removed at 5 min. intervals and cooled in cold running water. The colour reaction became visible after 5 mins. in the more concentrated samples, as a red ring at the base of the tube and after this the blue colour appeared. The colours were, therefore, read with two filters, Ilford spectrum green 604 and spectrum red 608, on the (Graph 12) Spekker absorptiometer. The blue component of the colour was the most stable and was found to be maximal after 15 mins. heating, in most cases. If heating was continued, the red component increased and purplish colours were produced. Although Josephson recommends heating for 20 minutes the optimal time for the

Graph 3



4

Graph 4

THE EFFECT OF HEAT ON THE COLOUR REACTION OF SODIUM TAUROGLYCOCHOLATE, FURFURAL, & 50% v/v SULPHURIC ACID.

Extinction

Ilford Spectrum red 608

Maximum transmission 6900

mg/100 ml

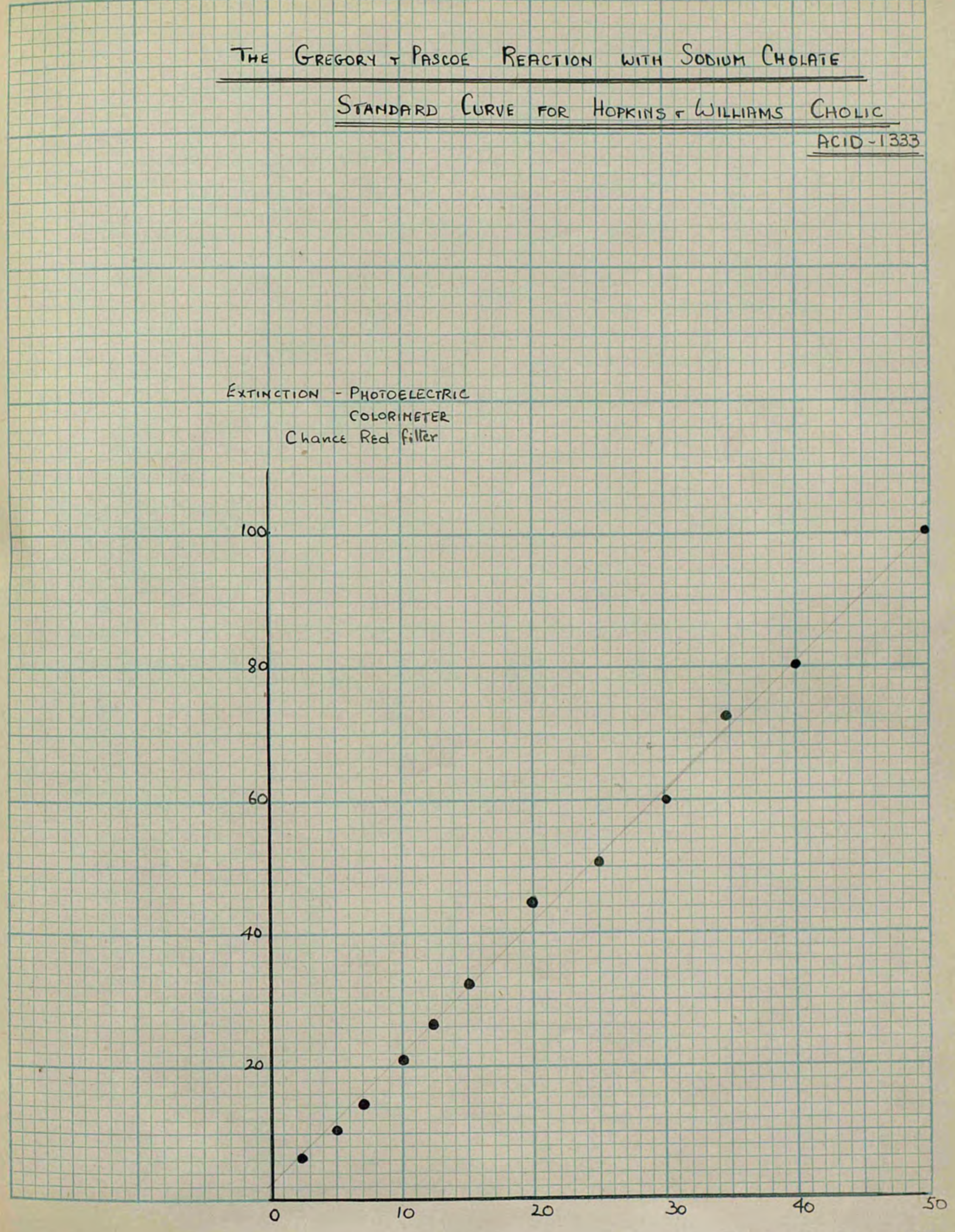
30
25
20
15
10
5
0

25
20
15
10
5

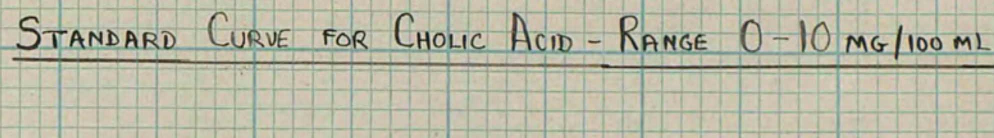
5 10 15 20 25

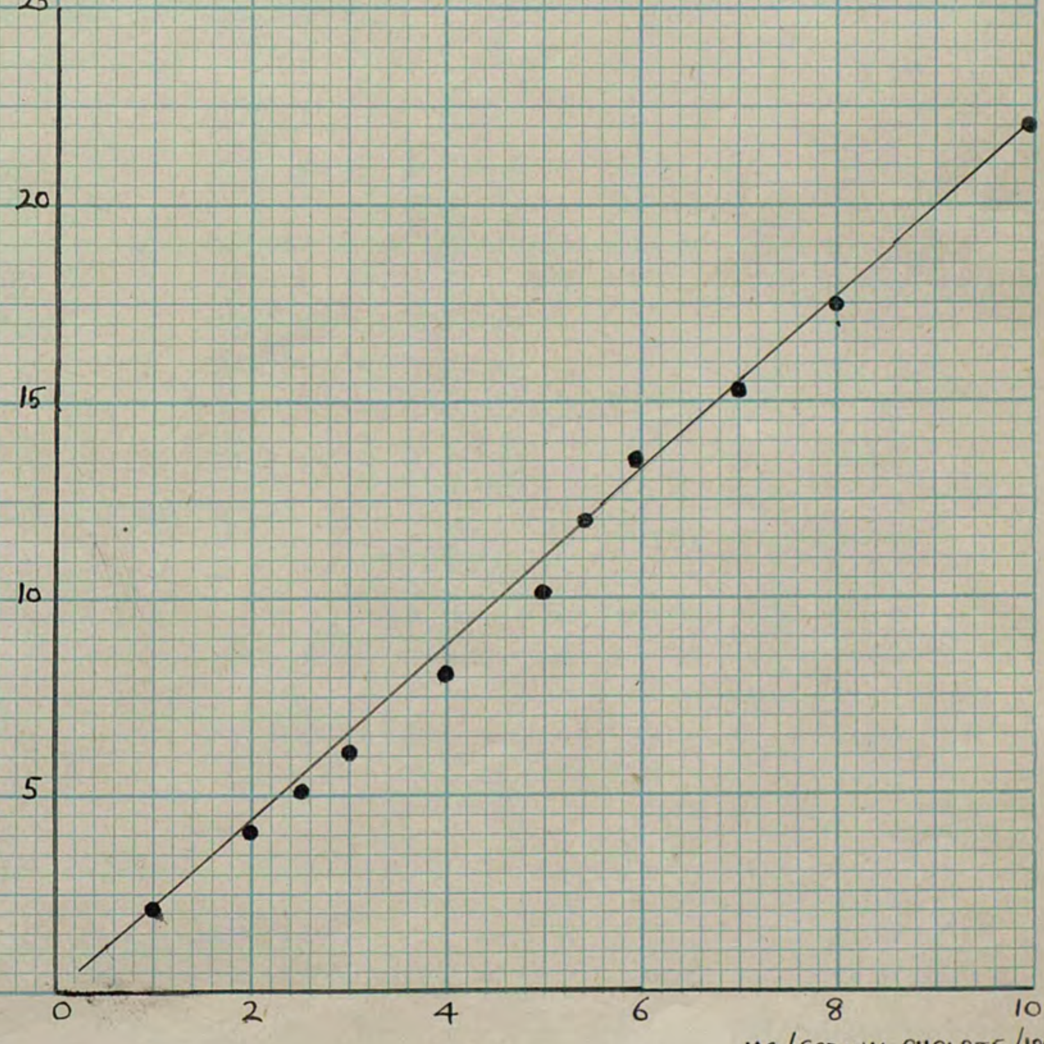
TIME (MINS)

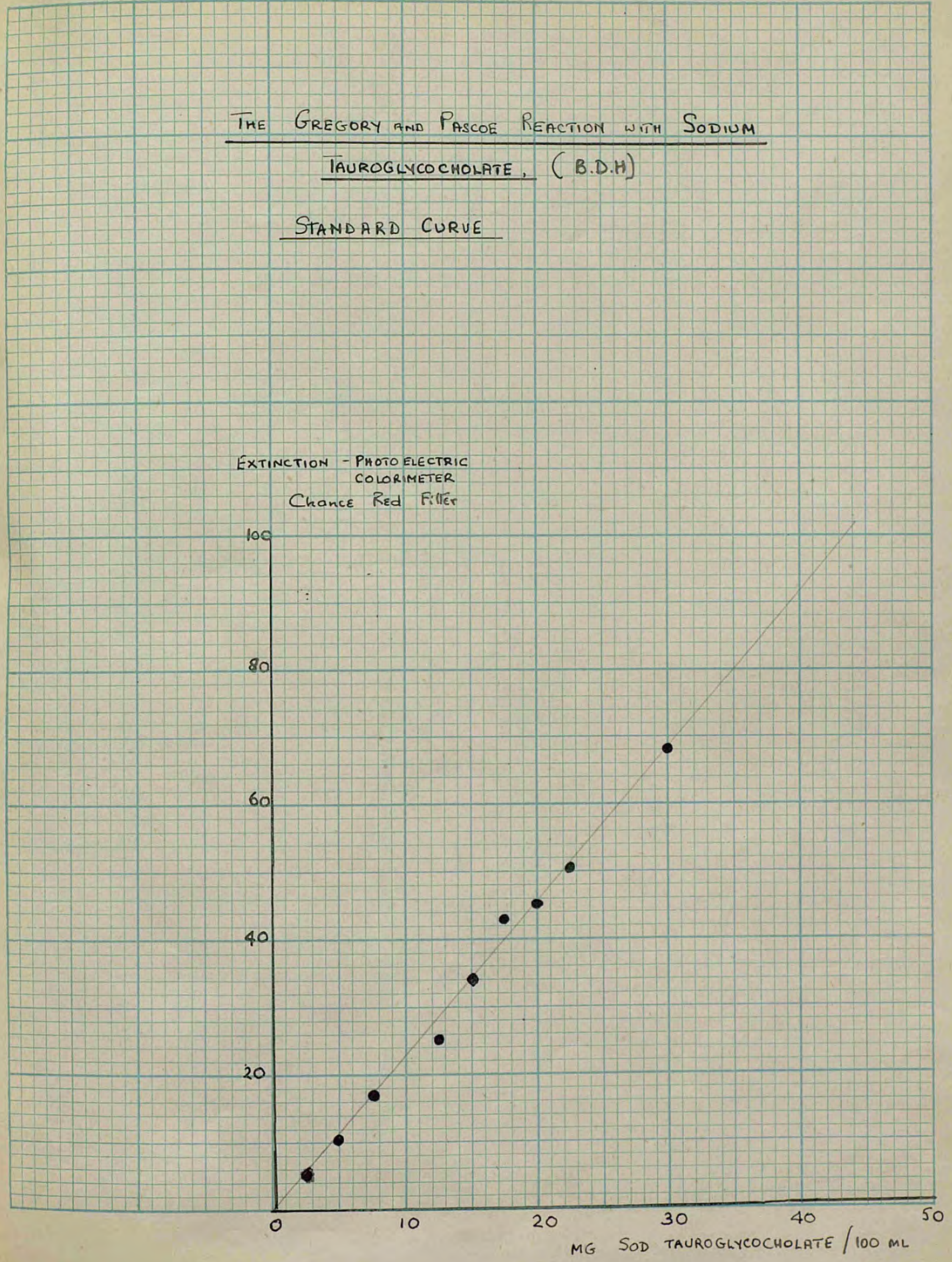
Graph 5



Graph 6


STANDARD CURVE FOR CHOLIC ACID - RANGE 0-10 MG/100 ML




THE GREGORY AND PASCOE REACTION WITH SODIUM
TAUROGLYCOCHOLATE, (B.D.H)
STANDARD CURVE
EXTINCTION - PHOTO ELECTRIC
COLORIMETER
Chance Red Filter
100
80
60
40
20
0
10
20
30
40
50
MG SOD TAUROGLYCOCHOLATE / 100 ML

development of the blue colour was taken as 15 minutes. This was repeated with solutions of sodium tauroglycocholate (B.D.H.) Graph 3 & 4.

Standard Curves

A standard curve was constructed for each fresh sample of cholic acid used for injection. Graph 5 shows a typical curve, given by Hopkins and Williams 1333 cholic acid. The curve for the range 0 - 10 mg./100 ml. is shown on a large scale in Graph. 6. In the working range this did not deviate significantly from a straight line. A commercial sample of bile salts was also checked in this way. Graph 7.

The Gregory & Pascoe reaction with other bile acids and derivatives. The compounds listed in Table 1 were kindly supplied by Dr. G.A.D. Haslewood, and tested by this reaction. Small portions were dissolved in glacial acetic acid (0.5 ml.) and the sulphuric acid and furfural added as above. The results are recorded as negative, weakly or strongly positive, and the shade of colour noted.

2) Application of the colour reaction to blood filtrates.

The original method (Josephson, 1935)

Precipitation of the proteins - 5 ml. of whole blood, without anticoagulant were taken into a flask containing 50 ml. of absolute ethanol and 2 ml. saturated barium hydroxide solution. A few grains of quartz sand were added, and the

TABLE 1.

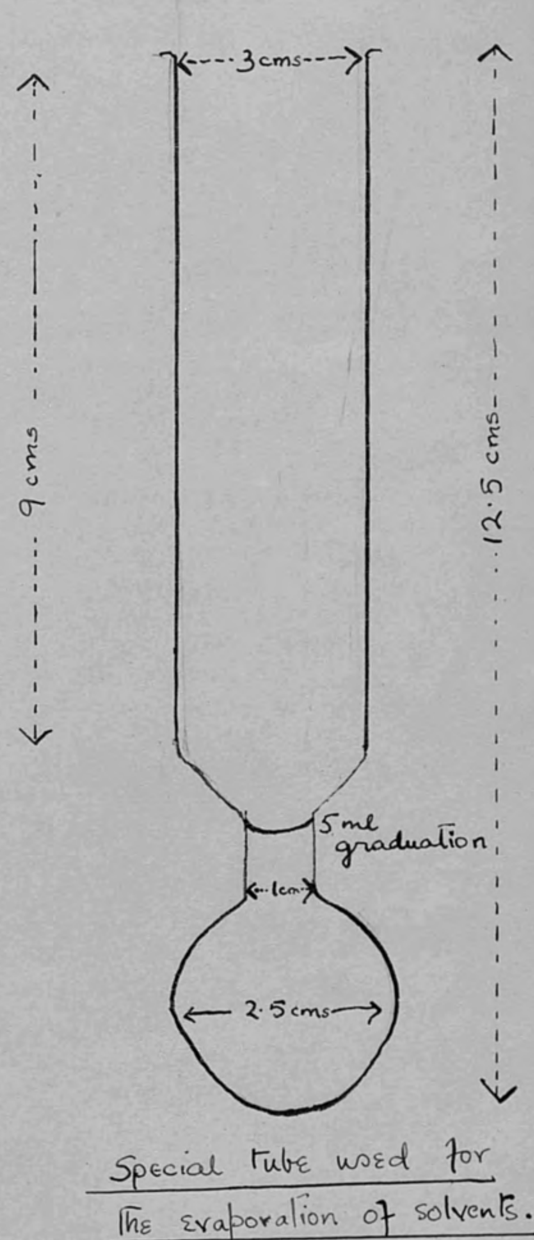
THE GREGORY & PASCOE REACTION WITH VARIOUS BILE ACIDS, DERIVATIVES, AND RELATED COMPOUNDS.

Compound	Formula	Reaction	Colour given
Ethyl cholate	OH; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; HO; OH	Strongly positive	Purple
Ethyl-3-keto-7:12 dihydroxycholanate.	OH; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; O; OH	Strongly positive	Blue
Ethyl-7-keto-3:12 dihydroxycholanate.	OH; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; HO; O	Strongly positive	Blue
Ethyl-12-keto-3:7 dihydroxycholanate.	O; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; HO; OH	Strongly positive	Blue
Ethyl-3:7-diketo-12-hydroxycholanate.	HO; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; O; O	Positive	Green
Ethyl-3:12-diketo-7-hydroxycholanate.	O; CH_3; CH_3 $CH(CH_2)_2COOC_2H_5$; CH_3; O; OH	Negative	-

Table 1. (continued)

Compound	Reaction	Colour
Hyodeoxycholic	Weak positive	Bluish
Scymnol	Strongly positive	Purple
Dehydro-androsterone	Strongly positive	Blue (dark)
Ethyl-7:12-diketo-3-hydroxy cholanate	Negative	—
Deoxycholic	Negative	—

fig. 1



Special tube used for the evaporation of solvents.

flask boiled for 5 minutes with shaking to ensure coagulation of the proteins. The volume was then made to the mark with neutral ethanol, and the flask left to stand overnight, until the supernatant liquid was clear. The volume was again adjusted, if necessary, and the proteins filtered off. The alcohol was passed through the same paper two or three times until perfectly clear.

Removal of excess barium - 0.3 ml. 2 N-H_2SO_4 was added, and the solution made alkaline to litmus with 0.5 ml. 2 N-NaOH. The solution was then filtered.

Evaporation of the solvent - Two 30 ml. portions were evaporated on an electric water bath, in the special tubes recommended by Josephson (Fig.1). When only 0.5 ml. remained, 4 ml. of water were added, and the tubes replaced in the water bath for 10 minutes, to dissolve the residue, and remove bile salts from the walls. The contents when cool were made alkaline with 0.5 ml. 2 N-NaOH.

Extraction of lipoids and cholesterol - 10 ml. neutral redistilled ethyl acetate were pipetted into each tube, which was then stoppered, and inverted several times. The solvent was separated from the aqueous layer by centrifuging for 15 minutes, and the aqueous fraction then made to the 5 ml. mark with water saturated with ethyl acetate. The tubes were shaken again and recentrifuged for 10 minutes.

Removal of the solvent - The ethyl acetate and the surface layer of insoluble material were removed by suction. The tubes were placed in a water bath and heated for 20 minutes to remove traces of solvent.

Preparation of the solution for colorimetric assay - The volume was made to the 5 ml. mark with water, and the solution filtered through a no. 45 Whatman paper. 2 ml. of the clear filtrate were taken for the colour reaction, as previously described. The values obtained by this method after the intravenous injection of 0.5 g. sodium cholate were investigated. A fasting blood sample, one 5 min. after the injection, and one 60 min. later, were taken and analyzed for cholates. In repeated experiments, the extinctions obtained were very low, and no blue colour was visible, even in the 5 min. specimen. The effect of giving 1.0 g. sodium cholate intravenously was therefore tried, and a bluish colour in the 5 min. specimen was obtained. Since none of these solutions gave satisfactory extinctions, it was decided to use 10 ml. blood for analysis and modify the analytical procedure.

3). The method of analysis finally adopted.

Precipitation of the proteins: 10 ml. of whole blood, without anticoagulant were pipetted with shaking into a 250 ml. Erlenmeyer flask, containing 85 ml. of a 60-40 ethanol-ether mixture and 5.0 ml. of saturated barium hydroxide solution,

with 0.4% barium acetate (sodium citrate or oxalate cannot be used as the barium would be precipitated). This mixture is sufficiently alkaline to retain the cholates in solution as the soluble barium salts, but does not convert the haemoglobin into alkaline haematin. The protein precipitate readily separates from the solvent, and the mixture can be filtered after one hour. From this 100 ml. 70 or 80 ml. of filtrate can be obtained by careful filtration with precautions to prevent evaporation of the solvents.

Removal of the excess barium: One drop of 5 N-sulphuric acid is added, followed by 2 drops 5 N-NaOH. By using small amounts of strong solutions a volume correction is avoided. The barium and sodium sulphates are filtered off after standing for at least 6 hours to ensure complete precipitation.

Evaporation of the solvent: A total of 40 ml. of the filtrate, equivalent to 4 ml. whole blood, are evaporated down in two 20 ml. portions. A current of compressed air is passed into each tube on the electric water bath, and this, together with the use of ether in the precipitating mixture, makes the evaporation quite rapid. The tubes used for this evaporation are shown in Fig. 1 Enough filtrate remains for a blank estimation - which is only necessary when large amounts of bilirubin are present, or for a repeat estimation.

Aqueous solution of the bile salts: 4.5 ml. of hot

distilled water containing a little alkali is used to dissolve the residue after evaporation. The sides of the tubes to which bile salts may adhere, are carefully washed down, using a long pasteur pipette, and the tubes replaced in the water bath for a further 10 mins.

Extraction of lipoid material: Each tube is well shaken with ethyl acetate as previously described. After the final centrifuging, the solvent and also the "collar" of insoluble solid material which has collected in the constricted portion of the tube, are removed by suction. The tubes are then heated on a boiling water bath for 20 mins. and the volume adjusted to the 5 ml. mark.

Filtration of the final solution: The solutions while still warm are filtered through a Whatman 45 paper. Occasionally a turbid sample is encountered, which can be cleared by adding 1 drop of glacial acetic acid, leaving to stand for an hour, and refiltering. 2.5 ml. of the filtrate (=2.0 ml. whole blood) are pipetted into a pyrex tube, and 5 ml. v/v H_2SO_4. 0.5 ml. of 2% furfural (freshly prepared) is added. The tubes are stoppered with cotton wool, and heated on a water bath for 15 min. at 65°C. The colours (after the tubes are cooled in running water) are read in a photo-electric colorimeter, with a Chance red filter, against a reagent blank prepared by substituting 2.5 ml. of water for the blood filtrate. The recovery of cholic acid from whole blood has

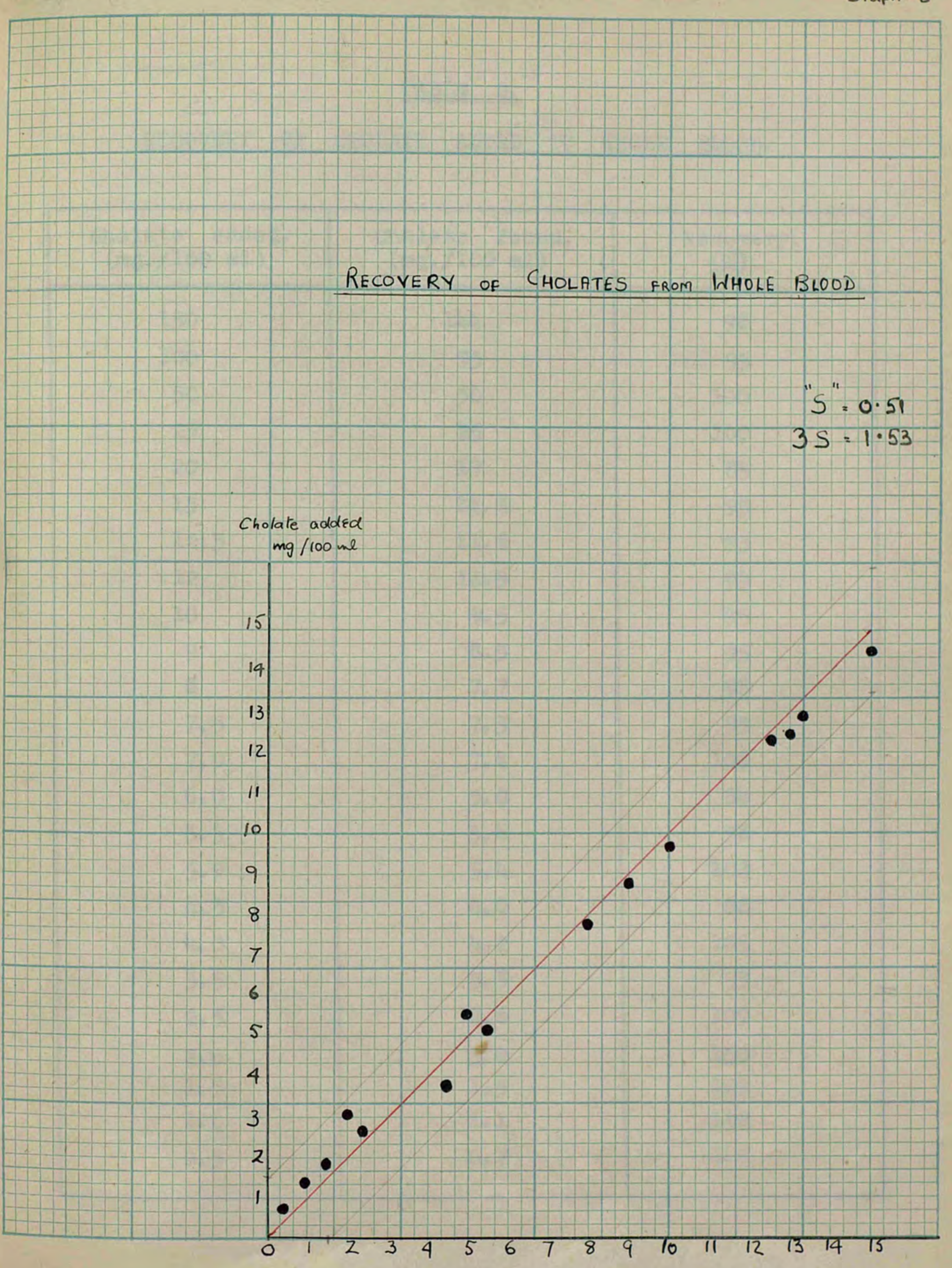

RECOVERY OF CHOLATES FROM WHOLE BLOOD
"S" = 0·51
3S = 1·53
Cholate added
mg /100 ml
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
CHOLATE FOUND. mg /100 ml

TABLE 2.

RECOVERY OF CHOLATE ADDED TO WHOLE BLOOD.

Cholate added. (mg./100 ml)	Cholate found. (mg./100 ml)	Recovery. (%)
150	144	96
100	95	95
50	50	100
25	25	100
20	17	85
15	14.3	95
12.5	11.6	93
12	11.9	99
10	9.5	95
9	8.5	95
8	7.7	98
7.5	7.3	95
7.0	7.4	106
6.0	5.0	84
5.5	5.4	98
5.0	5.4	108
4.5	4.0	88
4.0	4.2	105
3.5	3.3	93
2.5	2.6	109
2.0	3.0	150
1.5	1.5	100
1.0	1.1	110
0.5	0.7	140

been checked by an adequate number of analyses, many in duplicate. Table 2, Graph 8

The estimation of cholates in urine.

1. The colour reaction was tried on the diluted urine (Bothmann & Mann,[189] 1936) but very unsatisfactory results were obtained.

2. Removal of the pigments was tried by Dragendorf's method [256] (1865) and then with Norite at an alkaline pH. Various adsorbents such as alumina, Kaolin, Kieselguhr etc. were then investigated, but either the pigments were not removed, or added bile acids were also adsorbed.

3. Chromatography was also tried without success.

4. Oxidation of the pigments before their removal (Folin's method) was also tried.

5. Lichtman's method [257] (1938) gave unsatisfactory results.

6. Final method adopted.

a) for routine samples.

10 ml. urine made alkaline to phenolphalein with barium hydroxide (solid) warmed and filtered. This removed a large amount of the pigments and the cholates pass into the filtrate as soluble barium cholates. The excess barium is precipitated with 2 drops 5N H_2SO_4/alkaline with 3 drops 5N NaOH.

0.5 ml. filtrate for test.

0.5 ml. filtrate for blank.

With values of added cholic acid from 5 mg/100ml upwards, satisfactory results were obtained, only if a blank correction for heating urine with sulphuric acid was

introduced. However, with some heavily pigmented urines, which did not contain bile acids, some reaction occurred with H_2SO_4 and furfural, giving a blackish-brown colour. For this reason the results with this estimation cannot be considered highly accurate, but more in the nature of negative, slight positive, positive & strongly positive, depending on the green colour produced.

b) Quantitative estimation - by Soxhlet extraction.

This method is based on the work of Scott,[233] (1934). The urine is acidified with HCl, & saturated with ammonium sulphate and left to stand overnight. Bile acids, bilirubin & urinary pigments are precipitated. The precipitate is filtered off and washed with saturated ammonium sulphate. The filter paper is then thoroughly dried, cut up small, and extracted with absolute alcohol in a soxhlet thimble. Saturated barium hydroxide is added to the alcoholic extract, which is left overnight in the cold and the remaining pigments precipitated, and an almost colourless filtrate containing the bile acids is obtained. This is evaporated to dryness and the residue taken up in water for the colour reaction.

2. Material

a) Clinical

Subjects without liver disease were either members of the hospital staff or patients in hospital from some cause other than liver disease. The cases of liver disease were classified in give groups.

Clinical Material

		Number of Cases.	
		Resting level of blood cholates.	I.V. Cholic Acid tolerance
Group A.	Normal	50	16
Group B.	Obstructive Jaundice	17	16
Group C.	Cirrhosis	18 Inactive 6 Active 12	13 Inactive 5 Active 8
Group D.	Acute Hepatitis	50	29
Group E.	Secondary Malignant Disease	7	4
Group F.	Haemolytic Jaundice	6	5

b) The Histology of the liver Liver tissue was obtained by a modification of the aspiration liver biopsy technique of Iversen & Roholm[265] (1939). This has been described in detail by Sherlock[266] (1945). In a few cases, material obtained at necropsy or operation was used. These latter sources may be unreliable, since even before death, glycogen usually disappears from the liver cells and post mortem

autolytic changes, proceed very rapidly. (Van Beek & Haex, 1943). Necropsy often takes place some time after performance of the liver function test, and material obtained by surgical exposure of the liver is subject to the effects of trauma and anaesthesia. The interpretation of histological changes in small samples from the extreme liver edge is often difficult. The advantage of aspiration liver biopsy is that it can be done in close time relation to the liver function tests, and that fixation of the tissue is immediate.

In some of these cases the progress of the disease was studied by comparing sections obtained by serial biopsies with serial biochemical estimations.

Method of histological grading. Group B, Obstructive Jaundice. In every instance the common bile duct was completely obstructed, confirmed at operation or autopsy. Conspicuous liver changes were found in every case. Bile pigment was present in the canaliculi, especially centralobularly, sometimes precipitated to form the so-called "bile thrombi". Focal, bile stained necroses, usually in relation to the periphery of the lobules, were also seen. Fibrous tissue was increased in the portal tracts, with proliferation of the bile ducts. See Fig. 2 & 3. Section 6.

Group C. Cirrhosis of the liver. a) Latent. Cell damage is minimal and there are many bands of mature fibrous tissue, disrupting the normal architecture of the liver.

b) Active. Chronic diffuse liver disease with fibrosis, retrogressive parenchymal changes and regeneration of surviving cells. See Figs. 4 & 5. Section 6.

Group D. Acute Hepatitis. This includes simple infections, hepatitis and arsenotherapy jaundice. Histologically, the essential lesion is an acute inflammation with varying degrees of cell necrosis and the two types cannot be differentiated. The material has been grouped according to the extent of liver cell damage, in order to compare the histological changes with the chemical findings.

Histological grading in acute hepatitis

Grade.	Probable percentage of surviving liver cells.
A	75 - 100
B	50 - 75
C	25 - 50
D	less than 25

An example of each grade is shown. Figs. 6,7,8,9. Section 6.

Group E. Secondary malignant liver disease. In every instance the diagnosis and extent of hepatic involvement was confirmed at autopsy. Cases with jaundice due to occlusion of the common bile duct were excluded.

Group F. Haemolytic jaundice. A constant reticulocytosis was demonstrated in the peripheral block in every case and there was uroblinuria and hepatic siderosis.

Liver Function Tests.

1. The intravenous Galactose tolerance test.

2. The Hippuric acid synthesis test.

Biochemical estimations.

a) Serum. 1) bilirubin.
2) alkaline phosphatase.
3) total cholesterol.
4) proteins, total and differential.
5) colloidal gold reaction.

b) Urine. 1) bilirubin.
2) urobilurogen (Erhlich)
3) urobilin. (Ethanolic zinc acetate.)
4) bile salts. (Hay's test)

Details of these methods are given in Section 4.

Table 3

Class.	Normal A.	Obstructive Jaundice B.	Cirrhosis C.	Acute Hepatitis D.	Malignant E.	Haemolytic F.
No. of cases	50	17	18	50	7	6
Mean	1.6	3.7	1.8	2.8	2.1	1.5
S.D.	0.6	2.0	1.0	1.4	0.7	1.2

Table 4. Analysis of Variance

	Sum of squares	Degrees of Freedom	Mean Square	Variance Ratio	Fiducial Probability
Between classes	87.51	5	17.50	11.99	>0.01
Within classes	207.77	142	1.46	-	
Total	295.28	147	-	-	

3.

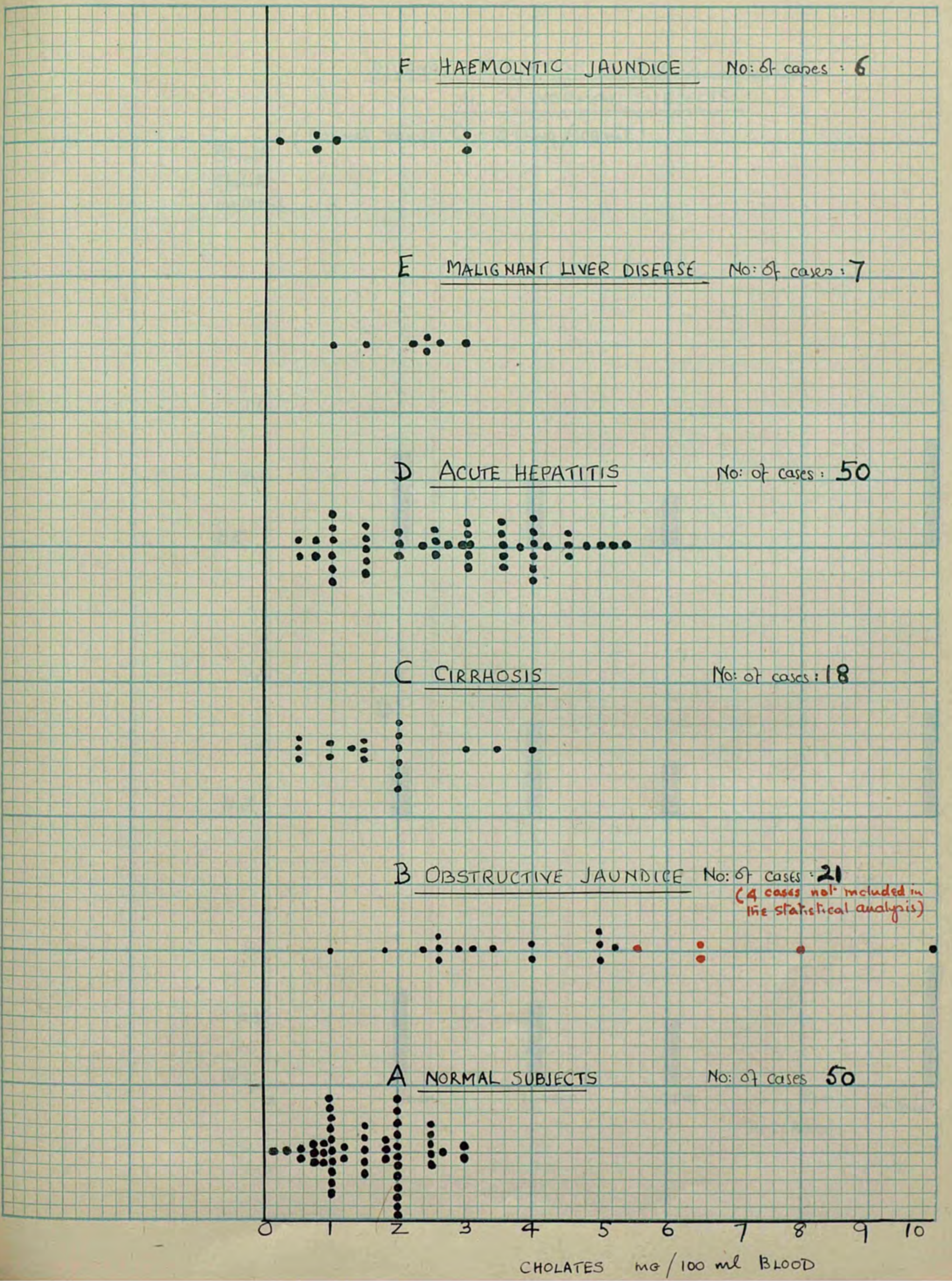
THE RESTING LEVEL OF BLOOD CHOLATES

The number of cases investigated was 148, classified as shown in Tables 3 & 4.

The standard deviation for any class is high, because of the wide scatter. For this reason the significance of the different levels for these classes showed that the differences found were highly significant. The fiducial probability was less than 0.01.

A dot diagram of the distribution is shown in Graph 9.

Graph 9



Graph 10

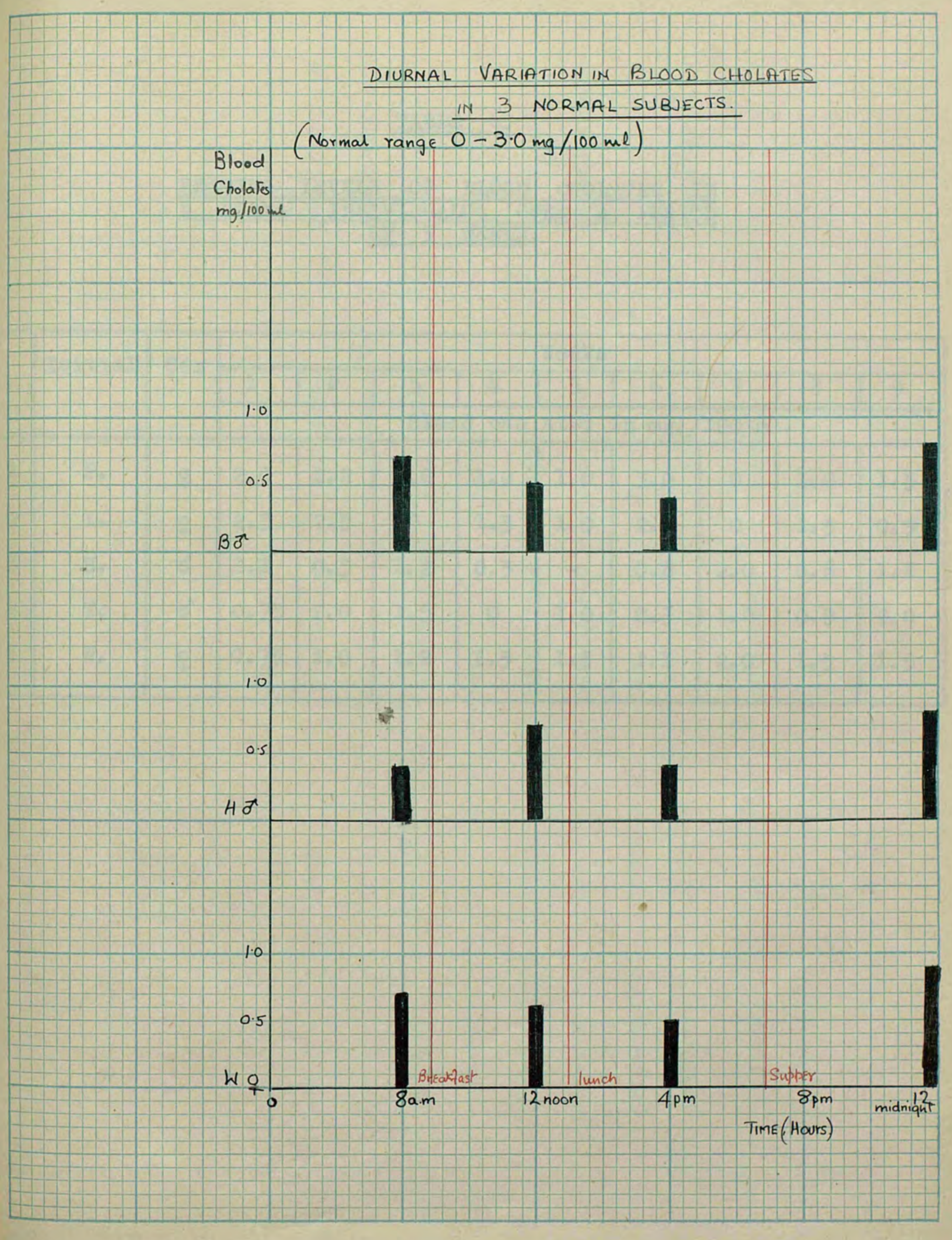
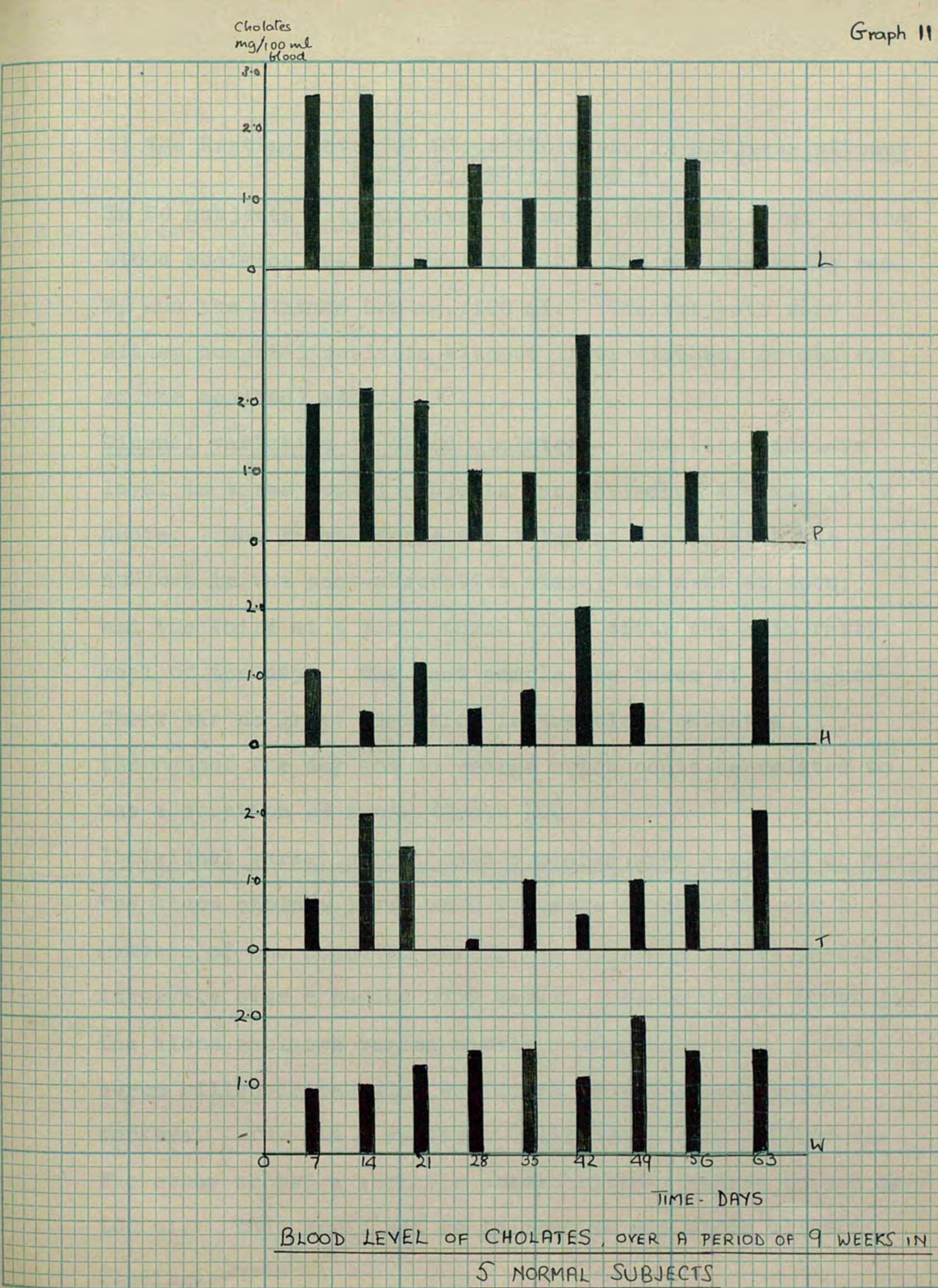


TABLE 6.

RESTING LEVELS OF BLOOD CHOLATES IN 5 NORMAL INDIVIDUALS, OVER A PERIOD OF 9 WEEKS.

	Initial	Sex	Weeks								
			1	2	3	4	5	6	7	8	9
			Blood cholates in mg./100 ml.								
1	P.	♀	2.0	2.2	2.0	1.0	1.0	3.0	0	1.0	1.5
2	L.	♀	2.5	2.5	0	1.5	1.0	2.5	0	1.5	0.9
3	H.	♂	1.1	0.5	1.2	0.5	-	0.8	2.0	0.6	1.8
4	T.	♂	0.75	2.0	1.5	0	1.0	0.5	1.0	0.9	2.0
5	W.	♀	0.75	1.0	1.2	1.5	1.5	1.1	2.0	1.5	1.5


Graph 11
Cholates mg/100 ml blood
3·0
2·0
1·0
0
L
P
H
T
W
0
7
14
21
28
35
42
49
56
63
TIME - DAYS
BLOOD LEVEL OF CHOLATES, OVER A PERIOD OF 9 WEEKS IN 5 NORMAL SUBJECTS

1) The Resting Level of Blood Cholates, in Subjects without Liver Disease. Group A.

In 50 subjects without liver disease, the mean level of the blood cholates was 1.6 mg./100 ml. The maximum value was 3, and the minimum 0.2 mg./100 ml. The subjects were either hospital patients without liver disease, or members of the hospital staff.

Diurnal variation in blood cholates:- The blood cholate level in three normal subjects was estimated 4 times in 12 hours. The first sample was taken at 8 a.m. with the subject in the post-absorptive state, the next at noon; and the third at 4 p.m., three hours after the main fatty meal of the day. The last sample was taken at midnight. There was no great variation in the blood cholates throughout the day and no change which could be related to the absorption of food. (Graph 10.)

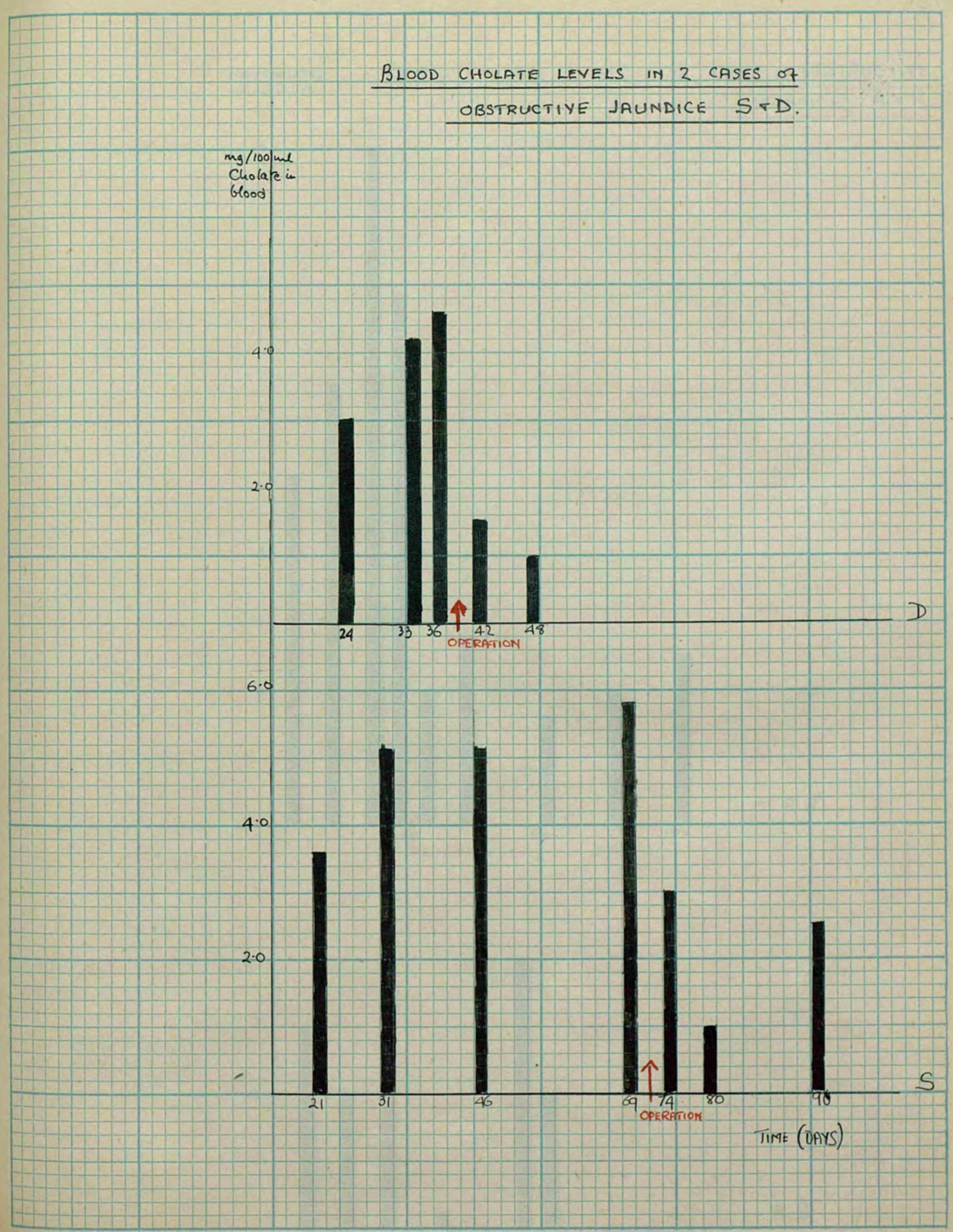
Individual variation in blood cholates over a period of months

The blood cholate level in 5 subjects (2 patients and 3 members of the hospital staff) was investigated at weekly intervals over a period of 9 weeks. (Table 6, and Graph 11.) The blood cholates varied widely during this time. The serum bilirubin was also estimated in the 3 laboratory workers. Two had a constant bilirubin of 0.5 mg./100 ml. The other showed a variable concentration, rising to 1.5 mg./100 ml. This could not be related to any other clinical finding.

TABLE 7.

BLOOD CHOLATES IN 4 CASES OF OBSTRUCTIVE JAUNDICE.
GROUP B.

Patient	Date	Diagnosis	Duration of jaundice in days.	Blood cholates mg./100 ml.	Serum Bilirubin mg./100 ml.	Serum Cholesterol mg./100 ml.
A. ♀	11.3.44.	Carcinoma of bile ducts	5	3.0	5.0	234
	17.3.44.		11	2.0	10.1	-
	23.3.44.		17	5.0	16.0	287
	13.4.44.		38	4.0	20.0	433
Death	15.4.44.		40	2.0	-	-
R. ♂	23.5.45.	Carcinoma of pancreas.	42	5.2	14.5	250
	31.5.45.		50	6.5	16.5	260
	6.6.45.		56	10.0	12.5	256
	8.6.45.		58	7.8	12.0	270
Cholecystotomy	14.6.45.		64	2.0	12.5	336
	21.6.45.		71	2.8	8.1	317
	5.7.45.		81	1.5	2.6	303
Cholecysgastrostomy.	17.7.45.		93	2.0	2.5	267
	27.7.45.		103	2.5	2.2	226
D. ♀	13.5.46.	Gallstones	24	3.0	11.0	287
	24.5.46.		33	4.2	9.0	-
	27.5.46.		36	4.5	5.6	261
Cholecystectomy	13.6.46.		42	1.5	2.2	437
	19.6.46.		48	1.0	1.7	358
S. ♂	14.11.46.	Carcinoma of ampulla of Vater.	21	3.5	4.0	147
	24.10.46.		31	5.1	5.1	282
	8.11.46.		46	5.1	5.7	243
	29.11.46.		69	5.8	7.2	185
Cholecystostomy.	4.12.46.		74	3.0	6.1	197
	10.10.46.		80	0.8	4.6	-
	24.12.46.		96	2.5		203
	6.1.47.		108	2.0		200


BLOOD CHOLATE LEVELS IN 2 CASES OF
OBSTRUCTIVE JAUNDICE S & D.
mg/100 ml
Cholate in
blood
4·0
2·0
D
24
33
36
42
48
OPERATION
6·0
4·0
2·0
S
21
31
46
69
74
80
96
OPERATION
TIME (DAYS)

Graph 13

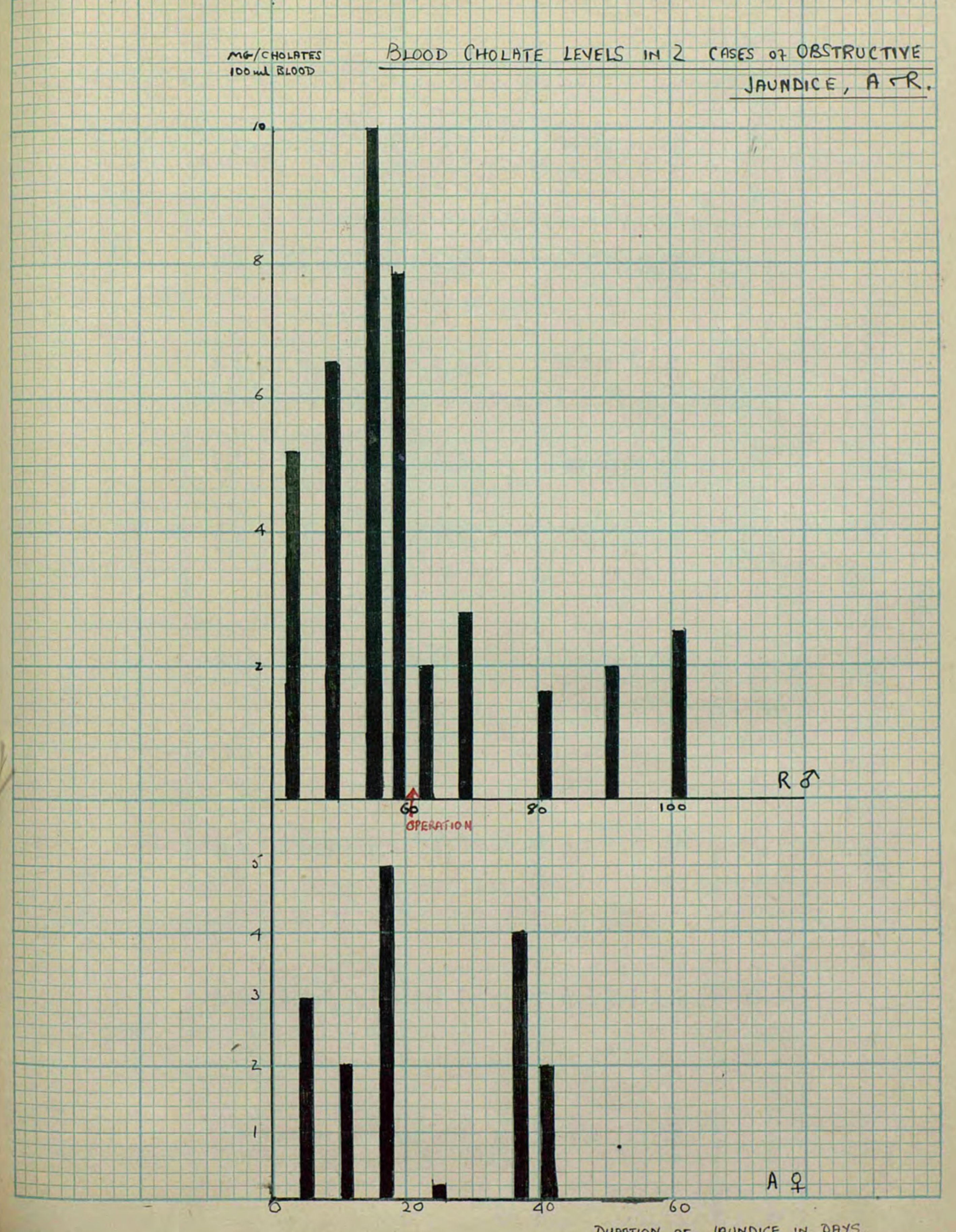


TABLE 8.

BILE ACID LEVEL AND GENERAL BIOCHEMICAL FINDINGS IN CIRRHOSIS (GROUP C).

I.	Inactive	mg/100 ml Bilirubin	U/100 ml. Phosphatase	mg./100 ml. Cholesterol	Proteins g./100 ml. Total	Alb.	Glob.	A/G	Coll.Gold	mg/100 ml. Bile Acid
	1	0.8	24	175	6.9	4.3	2.6	1.6	0	1.3
	2	0.5	4	178	6.8	4.8	2.0	2.4	0	1.5
	3	0.8	25	174	5.9	3.5	2.4	1.3	1	0.5
	4	0.7	12	185	7.0	5.2	1.8	2.8	0	2.0
	5	0.5	.9	167	6.0	3.1	2.9	1.1	0	1.0
	6	0.5	6.6	194	6.9	4.9	2.0	2.5	4	2.0
II.	Active									
	1	2.2	11	146	8.1	3.7	4.4	0.8	5	1.0
	2	0.9	15.4	180	5.1	2.3	2.8	0.9	5	2.0
	3	7.5	20	197	6.1	2.4	3.7	0.65	-	1.5
	4	1.7	15	256	7.0	2.8	4.2	0.7	-	3.5
	5	1.2	16.2	145	7.7	4.3	3.4	1.3	0	1.5
	6	2.9	27	-	6.2	3.3	2.9	1.1	-	2.0
	7	0.5	15	265	7.7	4.3	3.4	1.2	0	0.5
	8	5.8	11.1	169	4.6	2.4	2.2	1.1	5	2.0
	9	1.2	19	182	5.8	2.6	3.2	0.8	5	2.0
	10	12.2	21	-	6.9	2.8	4.1	0.7	5	3.0
	11	11.3	10.3	361	6.9	3.5	3.4	1.0	0	0.5
	12	3.4	10.2	176	6.1	2.0	4.1	0.5	-	4.0

2) The resting level of blood cholates in liver disease.

Group B. Obstructive Jaundice.

In 17 cases of obstructive jaundice the mean resting level (initial estimation) was 3.7 mg. cholates /100 ml. blood. The maximum value was 10 and the minimum 1.8.

Serial estimations were made in 4 of these cases. Table 7, Graph 12 & 13.

The fall in the blood cholate level after operation relieving the obstruction, is marked.

In this group the highest values are found, and the mean is the greatest in this series of 6 groups.

Group C. Cirrhosis

In 18 cases of cirrhosis of the liver, the mean resting level of blood cholates was 1.8 mg. /100 ml, the maximum was 4 and the minimum 0.5. This Group was divided into "active" and "latent" cirrhosis. In all the "latent" cases the blood cholate concentration was normal. Table 8, shows the bile acid level and the general biochemical findings in some of these cases.

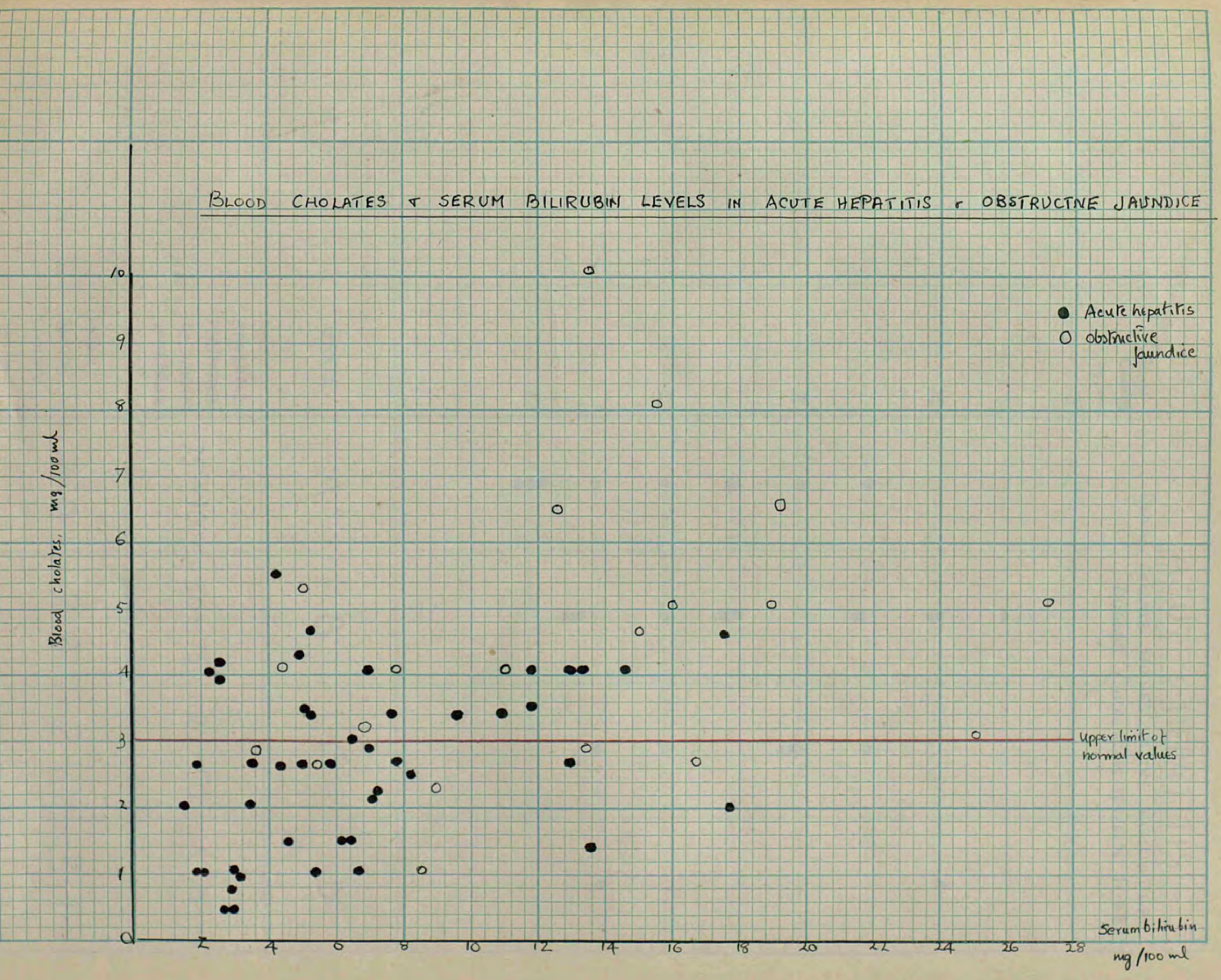
Group D. Acute Hepatitis

In the 50 cases of acute hepatitis studied, the mean resting level was 2.8 mg./100 ml.; the maximum 5.4 and the minimum 0.5.

TABLE 9.

CHOLATE LEVEL AND OTHER BIOCHEMICAL FINDINGS IN TWO CASES OF INFECTIOUS HEPATITIS

General biochemical findings.	Patient D.			Patient M.		
	mg./100 ml. blood.					
Bile acids	4.3	2.4	0	5.4	1.0	>0.5
Bilirubin	5.3	1.6	>0.5	4.2	1.5	0.9
Total Protein	7.8	7.0	7.5	8.3	7.4	8.1
Albumin	4.1	4.3	4.6	4.3	4.5	4.9
Globulin	3.7	2.7	2.9	4.0	2.9	3.0
Cholesterol	185	-	220	-	-	-
E.S.R. (mm.)	20	9	1	10	14	9.8
Colloidal Gold	0	0	0	5	4	3
Duration of jaundice in days	21	28	53	7	14	21



Graph 14

Graph 15

ACUTE HEPATITIS

BLOOD CHOLATE VALUES IN THE HISTOLOGICAL GRADES (INCREASING SEVERITY A→D)

Blood Cholates mg/100ml

5

4

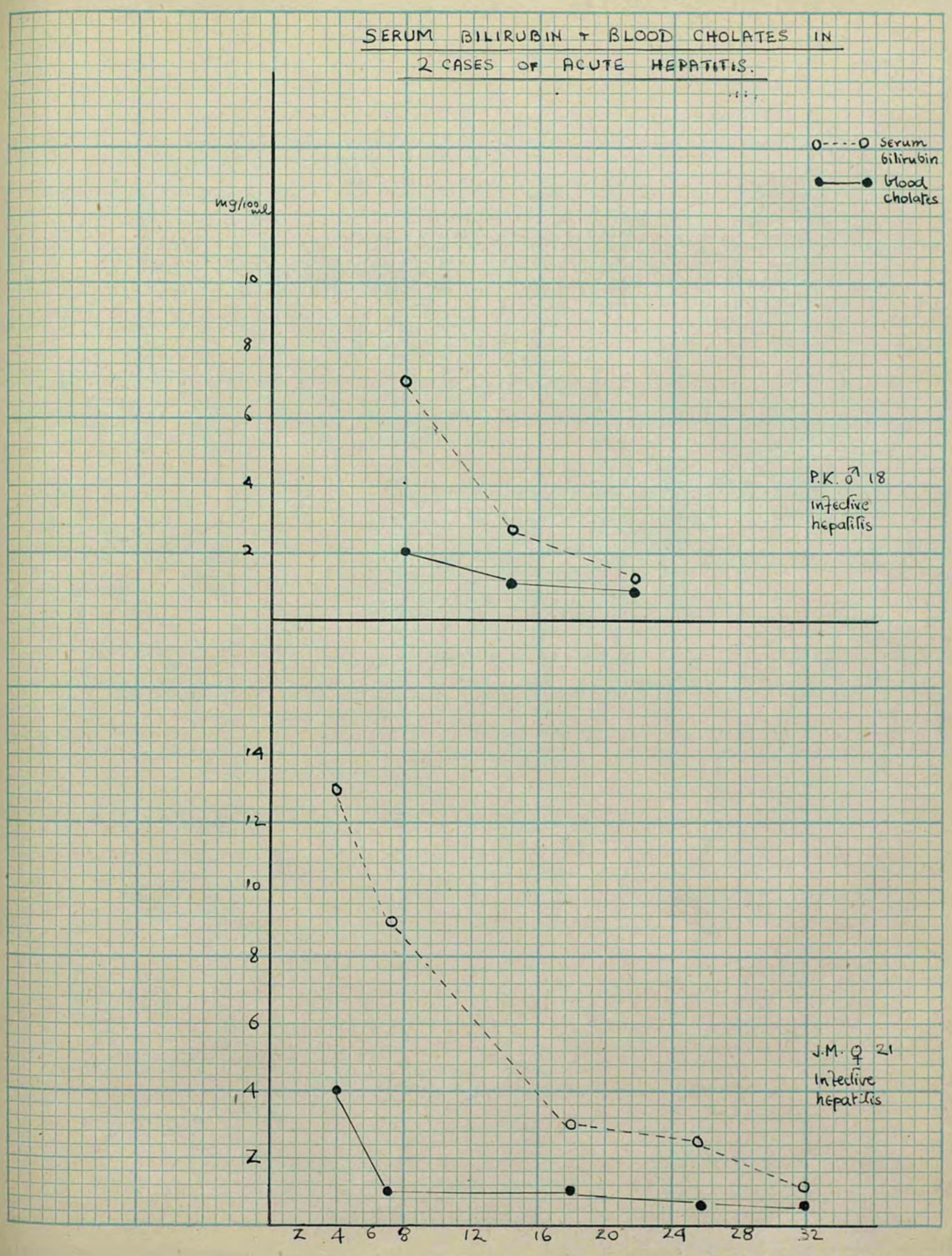
3

2

1

GRADE	A	B	C	D
PROBABLE PERCENTAGE OF SURVIVING LIVER CELLS	75–100	50–75	25–50	< 25
MEAN BLOOD CHOLATE LEVEL	2·0	2·8	2·6	3·1

Graph 16



The resting level of blood cholates was followed throughout the patients' stay in hospital in many cases, and related to the general biochemistry. The results in two typical cases are shown in Table 9. and in Graph 16.

There is considerable overlap in the values for blood cholates, obtained in Group B & D. Graph 14.

The blood cholate levels in the histological grades in acute hepatitis are shown in Graph 15.

Group E & F. Secondary Malignant liver disease and Haemolytic Jaundice.

The mean blood cholate level in Groups E & F was 2.1 and 1.5 respectively. The values found were within the limits for Group A, but the number of cases in both Group E & F is very small. The results are tabulated in Tables 10 & 11.

TABLE 10

BILE ACID LEVEL AND GENERAL BIOCHEMICAL FINDINGS IN SECONDARY MALIGNANT DISEASE. Group E.

Diagnosis	Urine	Serum								Blood
	Uro-bilin	Bilirubin mg/100ml.	Phosphatase U/100ml.	Cholesterol mg/100ml.	Total protein	Alb.	Glob.	A/G.	Coll. Gold	Cholates mg/100ml
1. Sec.Carcinoma Primary Stomach	+	0.5	4.7	147	5.4	4.4	1.0	4.4	0	1.5
2. Sec.Ca. Primary Colon	++	1.8	21	243	5.9	3.3	2.6	1.3	0	3.0
3. Sec.Ca. Primary Breast	++	0.5	13	165	5.3	3.2	2.1	1.5	1	2.5
4. Sec.Ca. Primary Stomach	++	0.8	24	-	6.7	3.7	3.0	1.2	-	2.4
5.	++	0.5	8.7	151	-	-	-	-	-	2.4
6. Sec.Ca. Primary Stomach	++	1.0	-	178	-	-	-	-	-	2.2
7. Sec.Ca. Primary Ethenoid	+	0.7	32	219	5.8	2.9	2.9	1.0	3	1.0

TABLE 11

BILE ACID LEVEL AND GENERAL BIOCHEMICAL FINDINGS IN HAEMOLYTIC JAUNDICE.
Group F.

Diagnosis	Urine	Serum								Blood
	Uro-bilin	Bilirubin mg/100ml.	Phosphatase U/100 ml.	Cholesterol mg/100ml.	Total protein	Alb.	Glob.	A/G.	Coll. Gold	Cholates mg/100ml.
1. Pernicious anaemia	++	1.1	12.6	-	-	-	-	-	-	1.0
2. Congenital acholuria	+	2.9	6.0	131	6.4	4.9	1.5	3.3	-	0.2
3. Pernicious anaemia	++	1.9	4.2	127	6.9	4.7	2.2	2.1	0	3.0
4. Post-trans-fusion jaundice	++	2.0	22	141	7.1	3.7	3.4	1.1	-	3.0
5. Pernicious anaemia	++	0.5	-	-	-	-	-	-	-	3.0
6. Pernicious anaemia	++	0.6	9	181	-	-	-	-	-	0.8

4. THE CHOLIC ACID TOLERANCE TEST

Technique of Test.

1). Preparation of the sodium cholate for injection. Pure cholic acid (Hopkins and Williams) was recrystalized from ethyl acetate, and dried in vacuo, m.p. 199°C. A 25% (w/v) aqueous solution of the sodium salt was made and sterilised by boiling. 4 ml. of this was equivalent to 1 g. sodium cholate.

Blood from the ante-cubital vein of the fasting subject is taken into a clean dry beaker, without any anti-coagulant, and 10 ml. of this immediately pipetted into a flask, containing the ethanol-ether-barium mixture, with shaking. 4 ml. of the sodium cholate is diluted to 20 ml. with saline and injected slowly. Provided a little time is taken over the injection, the patients do not complain of the characteristically bitter taste of the bile salts. Toxic effects have not so far been encountered. Thrombosis of the antecubital vein occurred inapproximately 5% of these cases, but it was never severe enough to need treatment. Blood samples (10 ml.) were taken 5 and 60 mins. after the injection, and in some cases at 120 min. The bladder was emptied before the injection, and all the urine passed at hourly intervals for 1, 2, 3, or 4 hours collected.

4.

2). A comparison of the intravenous cholic acid tolerance test, with other liver function tests, and the general biochemical findings.

A summary of the tests and estimations employed.

1. Liver function tests.

1) Intravenous Galactose tolerance. The galactose was prepared and sterilised as described by King, Harrison, and Delory,[268] (1940). The solution, (1 ml. 50% w/v, equivalent to 0.5 g. galactose/ g. body weight) was injected intravenously in the fasting subject. No toxic effects were encountered. Capillary blood, (0.2 ml.) was taken before the injection, 1/2, 1, 1½ & 2 hours later and washed into prepared tubes containing isotonic sodium sulphate (2.2 ml) and sodium tungstate (0.3 ml). The glucose in the blood was completely removed by fermentation with washed baker's yeast. The Galactose, which was not attacked, was estimated by the copper reduction method used for blood glucose. (King & Aitken,[269] 1940).

The 2 hour elimination test while able to distinguish between normal and abnormal responses could not differentiate between different types of normal tolerance curve. A modification of the "Galactose time" introduced by Barnes and King,[270] (1943) was used to express the different results given by normal and pathological cases.

Galactose time = $\frac{ax}{a-b}$ a = ½ hour blood galactose value
b = last figure for blood level before complete elimination.
x = 1/2, 1 or 1½ hr. figure, depending on value of b.

This expressed the rate of disappearance of galactose from the blood in minutes and approximately to an average rate of removal.

2) The intravenous Hippuric acid synthesis test. The synthesis of hippuric acid from benzoic acid and glycial has been widely used as a liver function test. We employed the intravenous modification of Quick, Ottenstein and Welbeck[271] (1938). 20 ml. of an 8.85 % solution of sodium benzoate, containing 1.77 g. (equivalent to 1.5 g. benzoic acid) was injected intravenously. The subject immediately emptied his bladder and was then given a pint of water to drink to ensure a sufficient urine volume at the hour. 60 minutes after the injection, the entire urine specimen was collected and sent to the laboratory. The urine was analysed by the method of Weichselbaum and Probstein[272] (1938). Dark specimens were decolourized with charcoal, and any specimen exceeding 150 ml. was evaporated down to about 100 ml. The hot urine was saturated with salt, and the filtrate audified with 50% sulphuric acid, until acid to congo red. The solution was left to stand overnight in the cold, and the cuptals of hippuric acid then filtered off by suction, dried, dissolved in water, and litrated with 0.5 N NaOH.

The normal range for excretion of hippuric acid under these conditions was found to be 0.75 - 1.21., hippuric acid expressed as sodium benzoate. (Sherlock,[273] 1946)

2. Biochemical Estimations.

1) Serum Bilirubin. This was estimated by the method of Haslewood & King[274] (1937). The serum was treated with diazotized sulphanilic acid and the direct Van den Bergh reaction recorded as positive or negative. The proteins were precipitated with saturated ammonium sulphate and the colour developed with 85% ethanol. The pink colour of the filtrate was compared with an artificial standard (Methyl Red) in a photo-electric colorimeter, using an Ilford tricolour green filter. The upper limit of normal was taken as 1.0 mg. bilirubin/100 ml. serum.

2) Serum alkaline phosphatase. A slightly modified King & Armstrong[275] (1934) method, was used. The amount of hydrolysis was determined when the serum alkaline phosphatase acted on a suitable substrate, in this case phenyl phosphate, at the optimum pH for 15 mins. The phenol liberated was estimated by the blue colour given by Folin & Ciocalteau's reagent, in a photo-electric colorimeter. (Ilford tricolour red filter.) 10 units / 100 ml. serum was taken as the upper limit of normal.

3) Serum Cholesterol. This was estimated by a modified Liebemann-Burchard reaction (Sackett,[276] 1925). Serum, 0.2 ml. was slowly dropped, with shaking into an alcohol-ether

mixture, which extracted the cholesterol and precipitated the serum proteins. The alcohol-ether was evaporated to dryness and the residue taken up in chloroform. The green colour which developed on treatment with acetic anhydride and concentrated sulphuric acid was compared with that given by a pure cholesterol standard, in a photoelectric colorimeter, using an Ilford tricolour red filter.

The normal range was taken as 120-230 mg. /100 ml. serum.

4) Serum Proteins. The Nesslerisation method of King, Haslewood, Delory & Beall (1937 & 1942)[277, 278] was used. The total proteins were estimated in serum diluted with isotonic sodium chloride. Another sample of the serum was treated with sodium sulphite solution to precipitate the globulins, and the filtrate used for albumin estimation. In both these cases the proteins were precipitated with zinc sulphate and sodium hydroxide and the precipitate digested with sulphuric acid containing selenium dioxide. The protein nitrogen was determined colorimetrically as ammonium sulphate, with Nessleis solution. The approximate protein values were obtained by multiplying the nitrogen figures by 6.25. The globulin was given by subtraction. The albumin/globulin ratio was also reported.

The normal ranges were taken as:-

Total protein	6-8g /100 ml.
Albumin	3.4 - 6 g./100 ml.
Globulin	1.5 - 3 g. /100 ml.
A/G ratio	1.3 - 4.

5) Serum Colloidal Gold. This was carried out according to Maclagen's[279] (1944) method. The colloidal gold was made up and stabilized with alkali (Patterson,[280] 1931). Barbitone buffer, (0.5 ml. pH 7.8) serum, (0.05 ml.) colloidal gold (2.5 ml.) were mixed in a Lange tube and allowed to stand overnight. The degree of precipitation was recorded, complete precipitation in any tube was reported as 5, none at all as 0. The intermediate degrees were interpolated by comparison.

6) Urinary Bilirubin - Methylene Blue Test. (Gellis & Stokes,[281] 1945). An aqueous solution of Methylene Blue (0.5%) was added dropwise, from a standard pipette to 5 ml. urine. The number of drops necessary to give a blue colour was noted. If more than 5 drops were needed, the urine was diluted with distilled water and the test repeated. If bilirubin is absent only 2 or 3 drops of Methylene Blue at most will be necessary. If bilirubin is present anything from 4 to 20 drops in the undiluted specimen will have to be added before a blue colour is obtained.

Hunter's test. The bile pigments were quantitatively precipitated from urine (8.0 ml.) with Barium Chloride (2 ml. 10%) (Pollack,[282] 1945). After centrifuging and washing twice with distilled water, this precipitate was treated with diazotized sulphanilic acid, the colour eluted with ethanol (95%), and a small amount of phosphate buffer added. A reddish brown colour was taken as a positive result. In

doubtful cases the test was repeated on larger quantities of urine, and the colour obtained was compared with the colour in a similar tube where the diazo reagent was replaced by hydrochloric acid. The results were reported as -ve, slight positive, positive and positive.

7) Urobilinogen. Erlich's aldehyde reagent (p-dimethyl animobenzaldehyde in strong Hcl) was added to a fresh sample of urine. A positive reaction was indicated by a cherry red colour.

The results were recorded as above.

8) Urobilin. The oxidation of urobilinogen to urobilin was completed by 0.1 N Iodine. Ethanolic zinc acetate was then added, and any fluorescence in the specimen viewed, after some hours against a dark background. The results were recorded in the same way.

9) Bile Salts. Hay's test was done under standard conditions. Urine (10 ml.) made acid to universal indicator with HCl was placed in a clean beaker, and held steadily at eye level in a good light. Resublimed sulphur was dropped gently onto its surface. Immediate sinking of the particles was reported as positive, whilst a few seconds delay in sinking indicated a slight positive. Most of the samples gave negative results. The results are shown in Table 12.

A critical evaluation of some of these biocehmical methods has been given by Sherlock (1946). In general, in acute hepatitis

TABLE 12.

THE INTRAVENOUS CHOLIC ACID TOLERANCE TEST COMPARED WITH THE GALACTOSE TOLERANCE, AND THE HIPPURIC ACID TEST OF LIVER FUNCTION.

Pt.	Diagnosis	Bili-rubin mg./100 ml.	Phos-phatase U/100 ml.	Chole-sterol mg./100 ml.	Galactose tolerance	G.T.	Hipp-uric acid gms.	Cholic acid Tolerance mg/100 ml.	Urinary cholates	Urinary uro-bilin
(1)	Obstructive Jaundice. Group B.									
A.	Carcinoma bile ducts.	5.0	48	234	55 32 2 0	67	0.7	2.0 11.0 7.5		-ve
T.	Carcinoma stomach.	10.7	67	189	87 - 58 34	149	-	1.8 10.0 4.8	+ve	-ve
R.	Carcinoma pancreas.	16.5	33	250	139 80 25 0	73	0.4	5.2 9.7 6.1	+ve	-ve
(2)	Cirrhosis. Group C.									
H.	Inactive.	0.8	24	175	40 18 0 0	-	0.5	2.0 7.0 1.8	-ve	-ve
Ha.	"	0.7	12	185	50 10 7 0	69	0.95	2.0 11.0 2.0	-ve	+ve
C.	"	0.5	40	105	122 10 0 0	33	1.05	1.5 6.8 1.3	-ve	
S.	Active.	2.2	11.1	146	138 59 55 20	111	o.3	1.0 6.5 3.2	-ve	+ve
(3)	Haemolytic Jaundice. Group F.									
J.	Haemolytic Anaemia	2.9	6.0	121	19 7 0 0	48	1.16	1.0 7.5 1.5	-ve	++ve
L.	P.A.	2.2	-	-	43 16 0 0	48	0.6	3.0 11.5 2.8	-ve	++ve
(4)	Secondary Carcinoma of Liver. Group E.									
T.		0.5	5.7	147			1.0	1.5 5.0 2.0	-ve	++ve
G.		0.5	11.0	165			0.3	3.0 8.7 4.0	+ve	++ve
Gy.		3.4	23	193	87 37 13 0	99	0.5	2.5 15.0 4.9	-ve	++ve

TABLE 12.
(Continued).

Pt.	Bili-rubin mg/100 ml.	Phos-phatase U/100 ml.	Chole-sterol mg./100 ml	Galactose tolerance				G.T.	Hippuric acid gms.	Cholic acid Tolerance mg./100 ml.			Urinary uro-bilin
				GROUP D. ACUTE HEPATITIS.									
Grade A.													
A.	3.0	22	178	50	5	2	0	63	1.1	0.2	7.5	0.2	+ve
S.	5.6	23	250	94	35	11	0	68	0.8	1.0	7.0	2.0	+ve
G.	2.2	17	111						0.95	0	6.0	5.1	+ve
Grade B.													
D.	3.5	25	206	136	88	45	16	102	0.71	2.5	9.0	4.9	+ve
G.	8.2	10	266	-	15	0	0	35	0.65	3.5	10	5.0	+ve
L.	6.4	20	256	73	59	0	0	60	0.5	2.5	14.8	4.8	+ve
M.	17.4	16	256	73	44	10	0	70	0.45	3.0	9.5	4.8	-ve
R.	2.5	33	-	77	32	25	9	104		1.0	5.5	2.5	+ve
V.	7.7	14.2	256	87	25	10	0	70	0.35	2.5	10.0	3.5	+ve
T.	12	12	190	71	50	10	0	68	0.35	2.5	5.3	3.0	-ve
M.	6.2	12	161	82	20	14	0	72	0.4	2.5	7.0	4.9	+ve
P.	18	12	186	102	47	26	19	105	0.4	0	5.7	4.5	-ve
Grade C.													
D.	7.8	16	200	117	68	3	0	65	0.43	1.0	6.3	4.8	+ve
H.	11.2	25	169	106	60	39	3	92	0.64	3.0	11.0	5.5	+ve
M.	11.5	17	152	91	60	40	4	94	0.63	-	-	4.8	+ve
Grade D.													
M.	15	17	217	113	55	31	8	97	0.41	3.0	7.0	5.5	+ve

the extent of the liver damage is reflected in the serum, bilirubin and serum albumin levels. Galactose tolerance and cholic acid tolerance are impaired only in the more severe grades. Impairment of hippuric acid synthesis is inconstant, and does not agree with the extent of the hepatic lesion.
No constant biochemical abnormalities are found in latent cirrhosis, but in active cirrhosis, abnormalities are demonstrated by all the methods used.
In obstructive jaundice, galactose tolerance and hippuric acid synthesis and cholic acid tolerance are abnormal and the serum proteins are decreased; the raised serum phosphatase is of use in diagnosis.
In the groups without jaundice, no decrease in liver function can be shown from an occasional rise in phosphatase, or low hippuric acid excretion.

Table 14. THE RATE OF DISAPPEARANCE OF INJECTED SODIUM CHOLATE FROM THE BLOOD OF 6 NORMAL SUBJECTS.

NO.	AGE	WT. (KG)	0.	5.	10.	15.	20.	25.	30.	35.	40.	45.	Disappearance Time (Mins)
1.	16	66	1.0	5.0	4.0	3.5	1.5	1.5	-	-	-	-	20
2.	56	67	1.5	8.5	-	4.5	3.5	3.2	1.0	-	-	-	30
3.	59	46	2.0	5.5	4.0	3.8	2.5	2.8.	2.0	2.0	2.0	-	30
4.	35	64	1.0	6.4	4.0	3.0	2.5	2.7	2.5	1.0	-	-	35
5.	45	51	1.0	5.0	3.0	2.5	2.7	2.0	1.8	-	1.0	-	40
6.	59	61	1.5	9.6	4.5	3.5	3.0	3.0	2.7	2.5	1.8	1.5	45

5.

1) CHOLIC ACID TOLERANCE IN a) SUBJECTS WITHOUT LIVER DISEASE

The results in 16 normal subjects are shown in Table 13.

Table 13.

CHOLIC ACID TOLERANCE IN 16 NORMAL SUBJECTS

	Minutes after injection			
	0	5	30	60
	Blood Cholates mg/100 ml.			
Average	1.5	6.7	2.6	1.6
Maximum	3.0	10.0	7.0	2.5
Minimum	1.0	2.5	1.0	1.0

In every case the blood level reached the pre-injection figure one hour later. The exact time at which the blood cholates were again normal was of interest, so this was more fully investigated in 6 subjects. Blood for analysis was taken at 5 min. intervals for 45 minutes after the injection. The results are shown in Table 14.

TABLE 15.

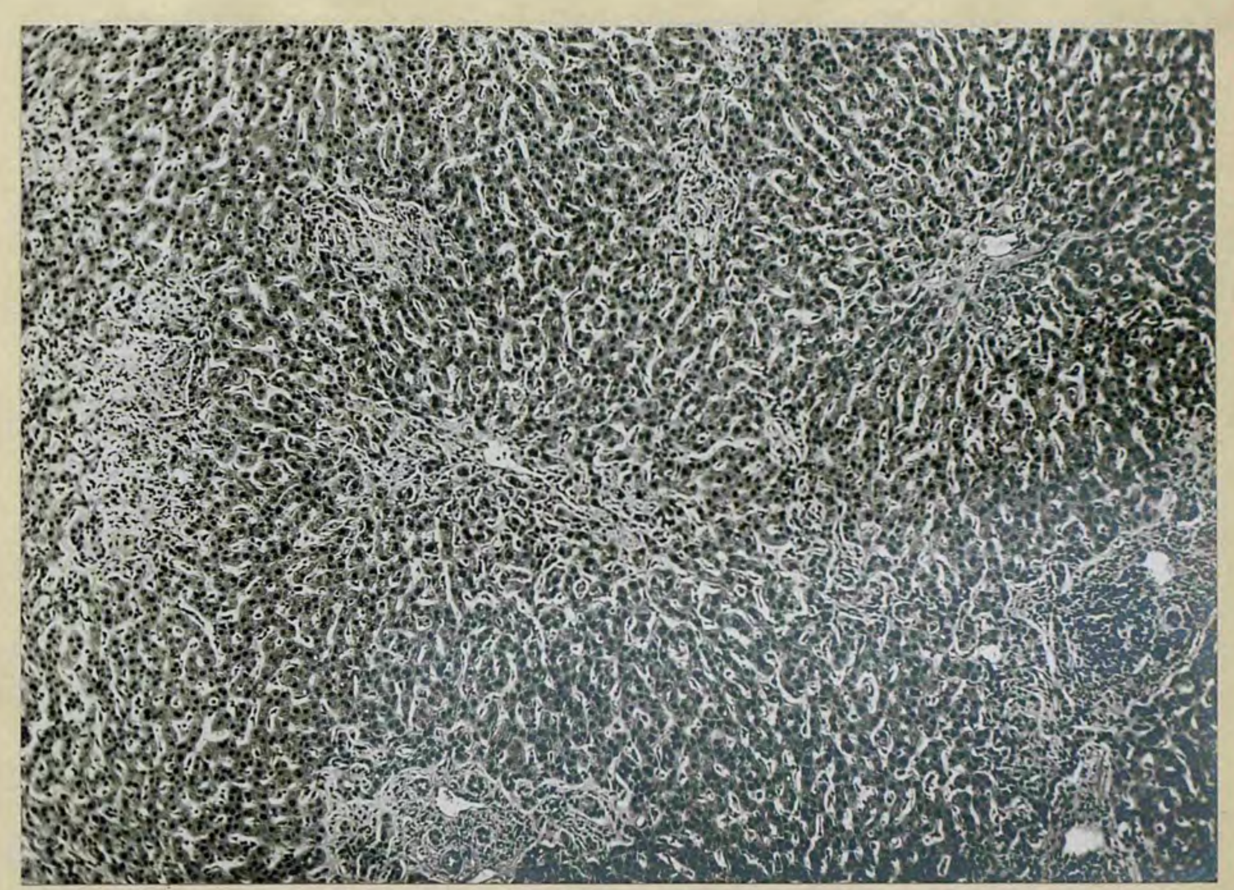
GROUP B. CHOLIC ACID TOLERANCE IN OBSTRUCTIVE JAUNDICE.

Pt.	Sex	Diagnosis	Duration of jaundice. (days)	Bilirubin (mg./100 ml.)	Cholesterol (mg./100 ml)	Blood cholate values (mg/100 ml) after injection				Urine Cholates after injection.
						Resting level	5 min.	60 min.	120 min.	
A.	♀	Carcinoma bile ducts	5	5.0	234	2.0	11.0	7.5	-	-
T.	♀	Carcinoma stomach (See jaundice).	63	11.0	189	1.8	10.0	4.8	-	+
L.	♀	" "	35	7.7	-	2.5	10.0	8.5	-	+
G.	♂	Ampullary carcinoma	42	9.1	-	2.3	6.2	5.0	-	+
J.	♂	Carcinoma pancreas.	28	15.0	182	5.0	13.0	7.0	-	+
B.	♂	Gallstones	42	20.0	163	4.5	10.3	8.5	-	+
AM.	♀	Carcinoma stomach (see jaundice).	23	34.0	265	3.3	7.3	3.5	3.7	+
S.	♂	Carcinoma head of pancreas.	7	13.0	-	2.9	5.8	4.7	4.0	+
SA.	♂	" "	3	4.3	-	4.0	11.5	7.2	4.8	-
R.	♂	Carcinoma pancreas.	63	14.5	250	5.2	9.7	6.1	5.8	+
To.	♂	Gallstones.		13.5	190	2.0	5.0	2.8	2.5	+

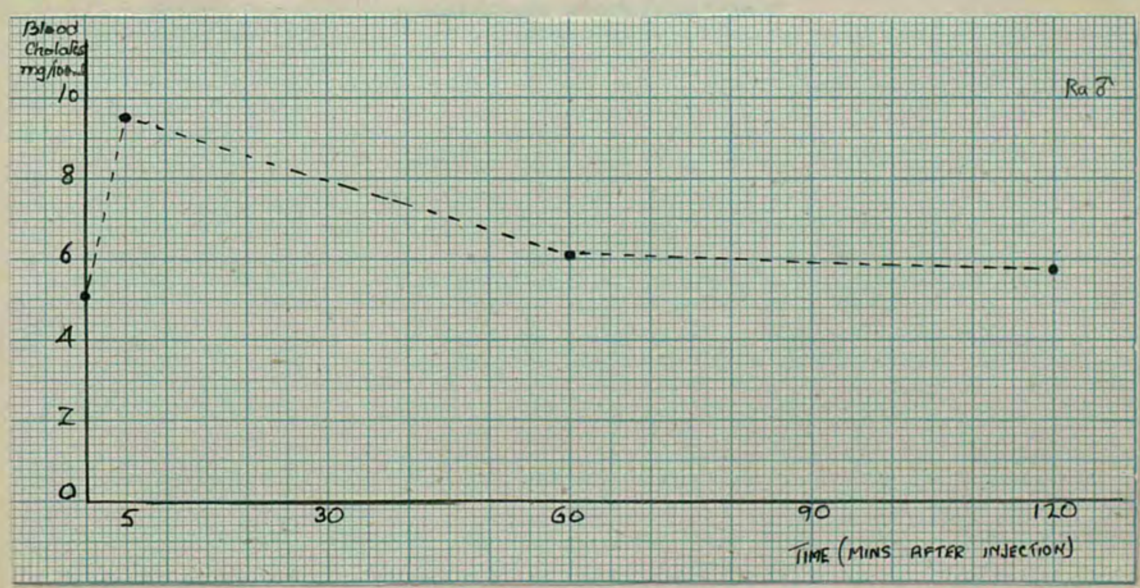
TABLE 15.
(Continued).

Pt.	Sex	Diagnosis	Duration of jaundice (days)	Bilirubin (mg./100 ml.)	Cholesterol (mg./100 ml)	Blood cholate values (mg/100 ml) after injection.				Urinary Cholates after injection.
						Resting level	5 min.	60 min.	120 min	
HE.	♀	Carcinoma bile ducts	100	19	872	6.5	8.7	7.6		++
SA.	♂	Carcinoma pancreas	47	5.7	243	5.1	10	9		++
TE.	♀	Carcinoma Ampullary	42	10.8	186	1.0	11	3.5		++
HA.	♀	Carcinoma pancreas	35	15.4	300	8.0	14.5	14.0		++

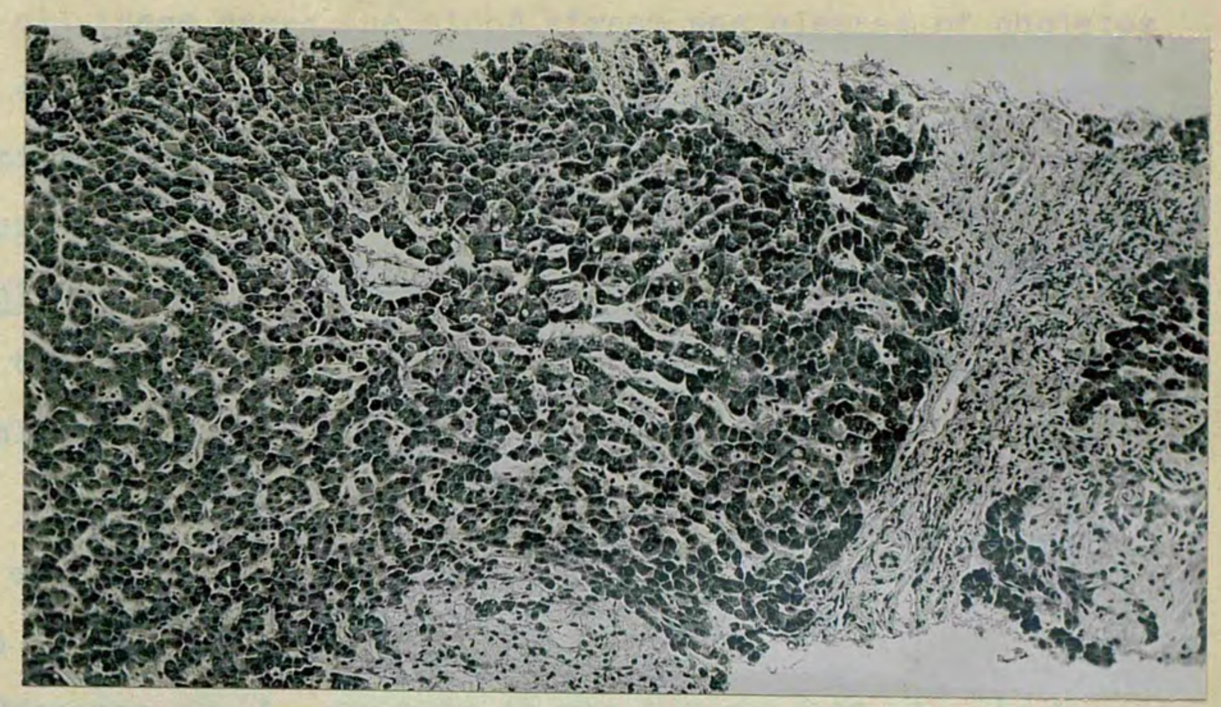
Group B. OBSTRUCTIVE JAUNDICE. Fig. 2.



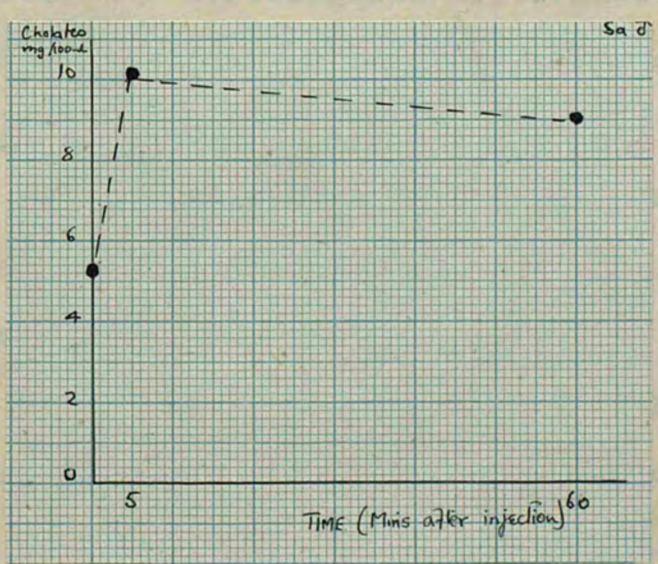
Intravenous cholic acid tolerance.



Group B. **OBSTRUCTIVE JAUNDICE.** Fig. 3.



Intravenous cholic acid tolerance.



In all these cases the blood stream was cleared of cholates by 45 mins. It was assumed that 60 mins. was the outside limit for clearance and when dealing with pathological cases, blood was taken before injection, and 5 & 60 mins. later.

<u>Cholates in the urine.</u> No cholates were found in the urine of these subjects after the injection of 1.0 gm. sodium cholate.

4. 2) A. <u>CHOLIC ACID TOLERANCE IN OBSTRUCTIVE JAUNDICE</u>

16 cases. In every case the blood cholate level at 60 mms. was higher than the initial level. In only one case was the difference small. Usually it was 3-5 mg./100 ml. In 5 cases a blood sample was taken 2 hours after the injection, and in each of these the cholate level was still raised.

Bile salts appeared in the urine after the injection in 13 out of the 16 cases.

Table 15.

Fig. 2 & 3 show the histological appearance of the liver, and the results of the intravenous cholic acid tolerance test.

B. <u>CIRRHOSIS</u>

In 13 cases of cirrhosis, the bile acid level 60 min. after the injection was not significantly raised in 5 cases. In only 3 cases was the 60 min. figure high. Bile acids appeared in the urine in 2 cases. Urobilin was present in all the urines. Table 16. Figs. 4 & 5 show the histological appearance of the liver in latent and active cirrhosis, and the results of the cholic acid tolerance tests.

TABLE 16.

CHOLIC ACID TOLERANCE IN 13 CASES OF CIRRHOSIS.

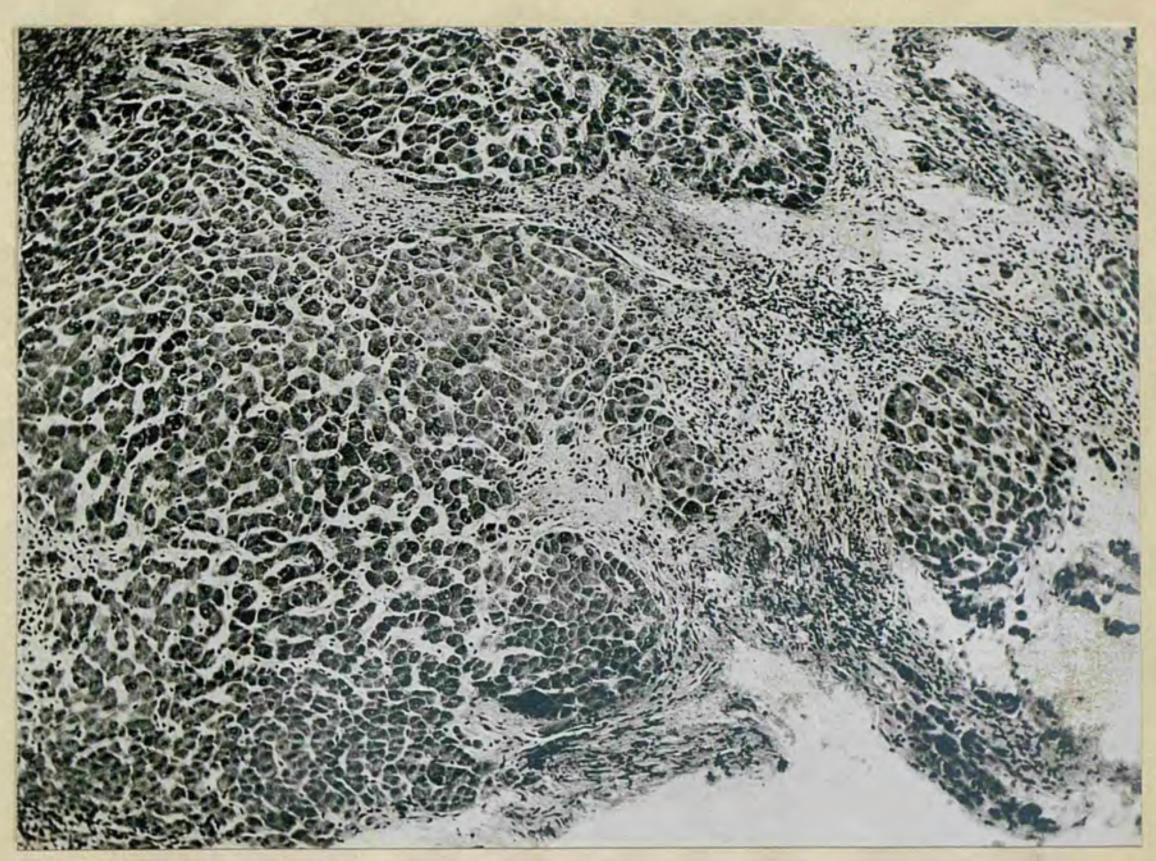
a. INACTIVE (5).

Patient		Bilirubin (mg./100 ml.)	Blood Cholates Fasting	5 min	60 min (mg./100 ml.)	Urinary Cholates (after injection)
H.	♂	0.7	2.0	11.0	2.0	-ve
C.	♂	0.5	1.5	6.8	1.3	-ve
B.	♀	0.8	0	4.5	2.0	+ve
JO.	♀	0.8	2.0	7.0	1.8	-ve
M.	♀	0.5	1.0	9.0	1.1	-ve

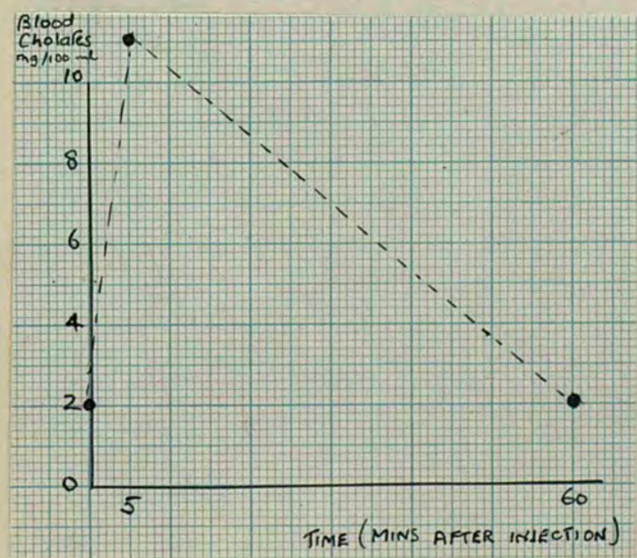
b. ACTIVE (6).

Patient		Bilirubin (mg./100 ml.)	Blood Cholates Fasting	5 min	60 min (mg./100 ml.)	Urinary Cholates (after injection)
S.	♂	2.2	1.0	6.5	3.2	-ve
R.	♂	0.9	2.0	14.5	3.5	+ve
M.	♂	7.5	1.5	3.9	3.5	-ve
M.	♀	1.7	3.5	8.8	6.0	++ve
K.	♂	2.1	2.0	8.0	2.5	-ve
W.	♀	1.2	1.5	9.0	4.0	-ve
I.	♂		2.5	6.0	2.5	-ve
H.	♂		0.5	5.0	2.0	-ve

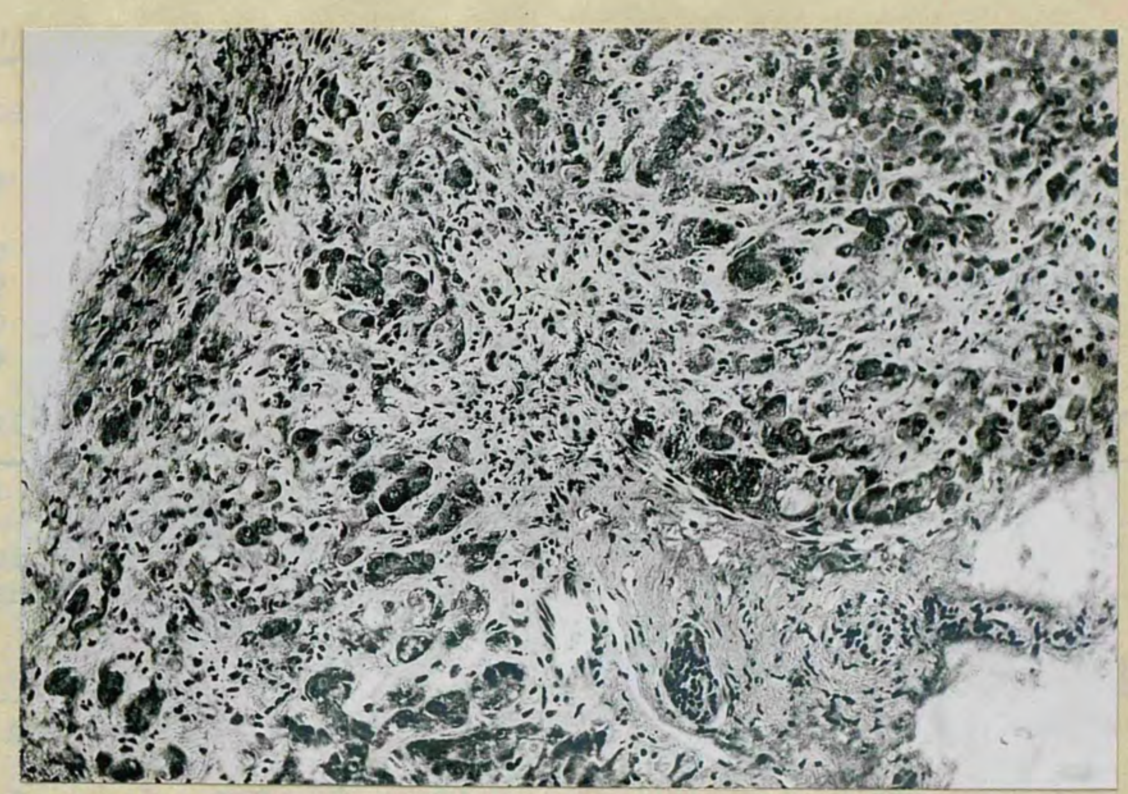
Group C. LATENT CIRRHOSIS. Fig. 4.



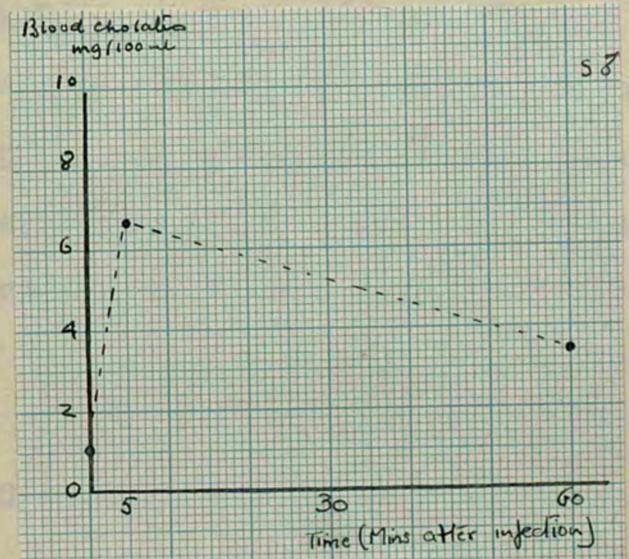
Intravenous cholic acid tolerance.



Group C. <u>ACTIVE CIRRHOSIS.</u> Fig. 5.



Intravenous cholic acid tolerance.



6. ACUTE HEPATITIS

No. of cases investigated, 29.

These were classified according to their histological grading.

Group A, 7 cases.
Group B, 13 cases.
Group C, 6 cases.
Group D, 3 cases.

The composite results for these groups are shown in Table 17.

Table 17. Cholic Acid tolerance in acute hepatitis

Histological grade	Resting Level	Blood cholate level (mg/100ml) 5 min specimen	60 min. specimen.	No. of cases.	No. of normal responses
A	1.4	8	3.1	7	3
B	2.7	11	4.6	13	1
C	1.8	8.3	4.7	6	1
D	2.8	8.7	4.2	3	0

A similar response was obtained in the cases of obstructive jaundice studied. There was no difference in the type of curves obtained.

Table 18 shows the individual results. Urobilin is present in the urine in Group A, and in 3 out of these 7 cases, cholates appeared in the urine after the injection. Urobilinuria occurs in 7 out of 13 cases in Group B, while cholates are present in 10 cases. In Group C and D absence of urobilin is paralleled by the excretion of cholates.

In Group C and D, urobilin is absent, and cholates present, with one exception in Group C. With increasing histological damage, more

TABLE 18.

INTRAVENOUS CHOLIC ACID TOLERANCE IN ACUTE HEPATITIS

Pt.	Sex	Diagnosis	Histo-logical grade. *	Dura-tion of jaun-dice. (days)	Serum Bili-rubin mg./100 ml.	Blood cholates (mg./100 ml.) Mins. after injection.				Urinary Cholates	Urinary uro-bilin
						Resting level	5	60	120		
B.	♂	Arseno-therapy jaundice	A	8	2.5	1.0	6.0	2.2	-	0	++ve
G.	♂	Infectious hepatitis	A	8	0.5	5.0	6.0	5.1	-	23	++ve
S.	♂	" "	A	9	5.4	1.0	7.0	2.3	-	33	++ve
N.	♂	" "	A	21	7.7	4.5	22.0	6.0	-	0	++ve
Be.	♂	" "	A	Not Jaun-diced	1.0	1.5	5.0	1.5	-	0	++ve
M.	♂	" "	A	2	1.6	2.0	6.0	2.5	-	5	++ve
K.	♂	" "		8	7.2	2.0	5.0	1.9	-	0	++ve
Mu.	♂	Arseno-therapy jaundice	B	10	6.2	2.5	7.0	4.9	-	80	-ve
T.	♂	" "	B	14	12.0	3.3	9.5	4.3	5.0	0	-ve
D.	♂	" "	B	4	3.5	2.5	8.0	4.9	-	26	-ve
L.	♂	" "	B	15	6.4	1.5	14.8	4.8	3.0	31	-ve
De.	♂	" "	B	22	7.7	1.5	17.0	3.8	2.5	13	++ve
R.	♂	" "	B	9	1.8	2.5	13.5	5.5.	-	100	++VE
O'K	♂	" "	B	13	6.5	1.5	15.0	4.0	-	22	-ve
V.	♂	" "	B	29	7.0	2.5	10.0	4.8	3.5	24	++ve

TABLE 18.
(Continued)

Pt.	Sex	Diagnosis	Histo-logical grade.	Dura-tion of jaun-dice. (days)	Serum bili-rubin mg./100 ml.	Blood cholates (mg./100 ml.) mins. after injection.				Urinary cho-lates	Urinary uro-bilin
						Resting level	5	60	120		
Du.	♂	Arseno-therapy jaundice	B	7	7.8	1.5	10.0	2.0	2.0	0	++ve
Ma.	♀	Infectious hepatitis	B	10	14.6	4.8	10.0	6.4	-	0	++ve
Gr	♂	" "	B	14	8.2	3.5	10.0	5.0	-	23	++ve
F.	♂	" "	B	3	4.4	4.0	6.5	5.0	-	36	++ve
W.	♂	" "	B	2	6.3	3.0	9.0	5.0	-	10	
H.	♂	Arseno-therapy jaundice	C	13	11.2	3.0	11.0	5.8	4.8	30	-ve
He	♂	" "	C	7	14.5	2.8	6.8	4.5	4.3	12	-ve
Me	♂	" "	C	9	12.0	3.0	6.5	4.8	-	20	-ve
A	♂	" "	C	7	14.0	0	7.5	0.5	-	12	-ve
SP.	♂	Infectious hepatitis	C	15	11.2	1.0	9.0	8.5	-	11	-ve
P	♂	" "	C	13	17.6	1.0	5.7	4.5	34	0	++ve
Ma.	♂	Arseno-therapy jaundice	D	1.0	14.6	4.8	10.0	6.4	-	45	-ve
P.	♂	" "	D	14	7.3	0.5	7.2	1.3	-	24	-ve
C.	♂	Infectious hepatitis	D			3.0	9.0	5.0	-	57	-ve

cholates are excreted in the urine which is to be expected.
Figs. 6, 7, 8 & 9 show the histological appearance of the liver in each grade of hepatitis and the corresponding cholic acid tolerance test.

In 7 acute cases, the cholic acid tolerance test was repeated during the course of the hepatitis, until clinical recovery with a serum bilirubin of less than 2 mg/100 ml. had occurred. Table 17 shows one such case, a severe arsenotherapy jaundice of 10 days duration. Figs. 9, 10 & 11 give the histological appearance of the liver.

Table 19. Cholic acid tolerance during hepatitis & recovery

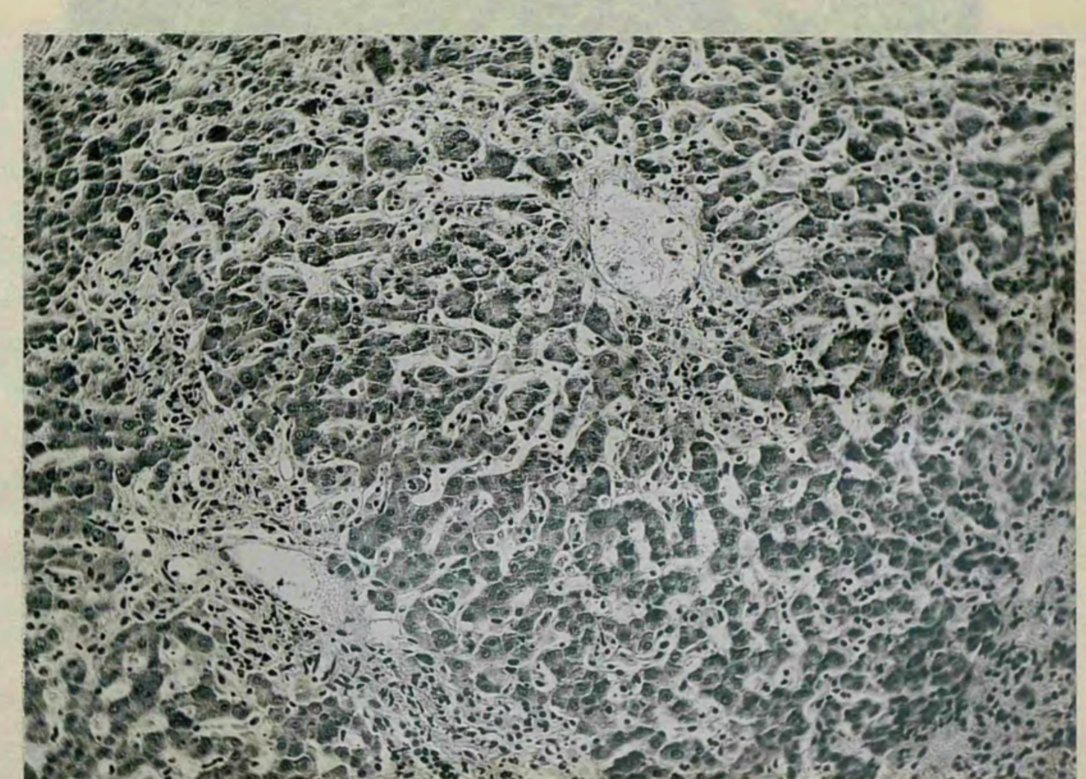
	Liver biopsy 1	Liver biopsy 2	Liver biopsy 3
Duration of jaundice in days	10	22	33
Serum bilirubin mg/100 ml.	14.6	14	2.6
Blood cholates mg/100 ml.			
1) resting level	4.8	10	6.4
2) 5 min. specimen	3	7	5.5
3) 60 min. specimen	1.0	10	1.0
Bile salts excreted in urine up to 4 hours after the injection.	96 mg.	85 mg.	0

D. SECONDARY CARCINOMA OF LIVER

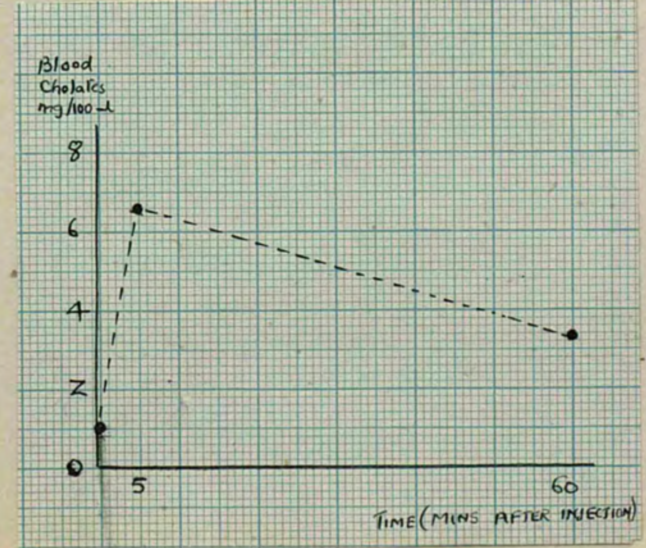
Table 20 shows the results of cholic acid tolerance in the 4 cases studied: in 2 cases the tolerance was abnormal and in one of these, cholates appeared in the urine.

Group D. ACUTE HEPATITIS. Fig. 6.

Histological Grade A.

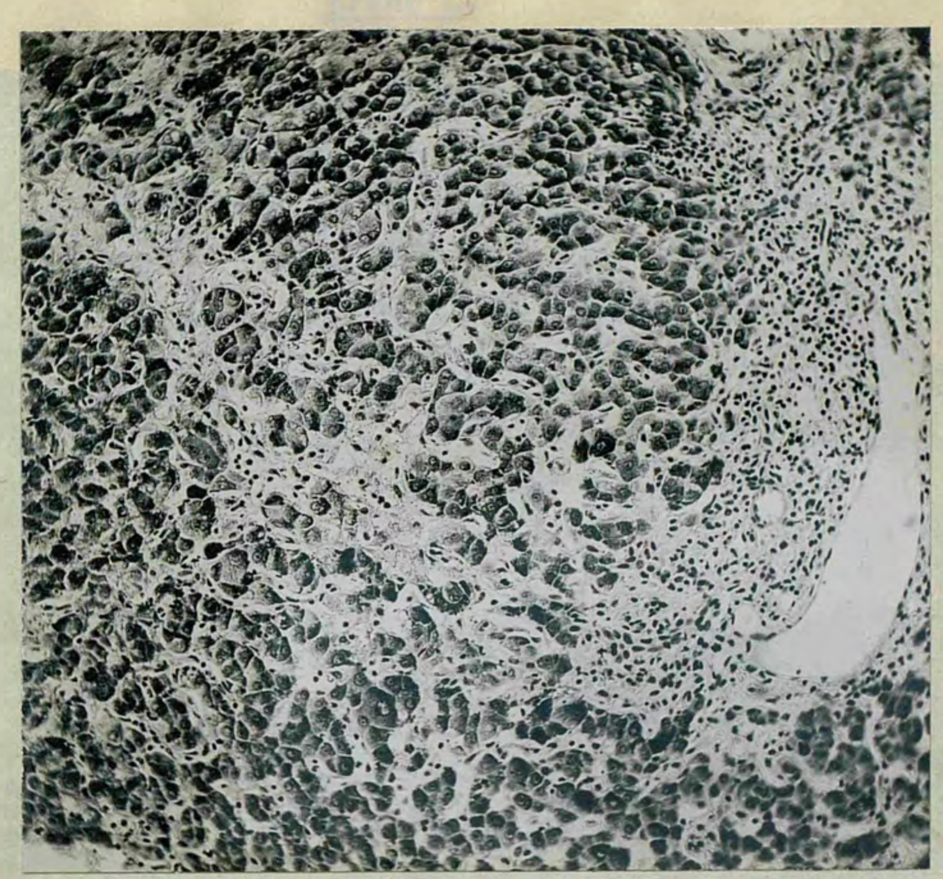


Intravenous cholic acid tolerance.

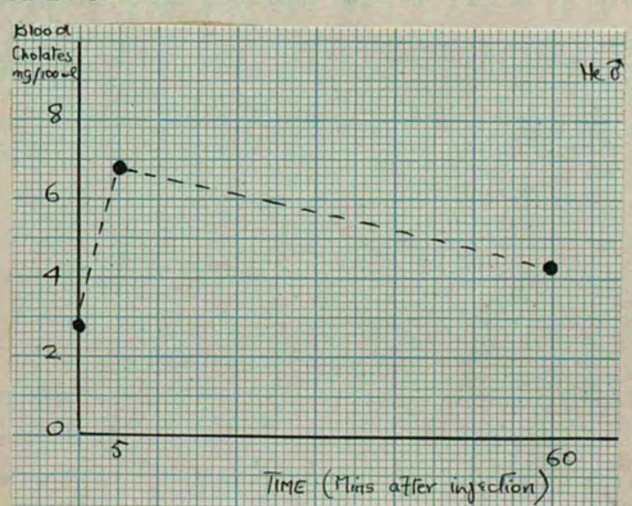


Group D. ACUTE HEPATITIS. Fig. 7.

Grade B.

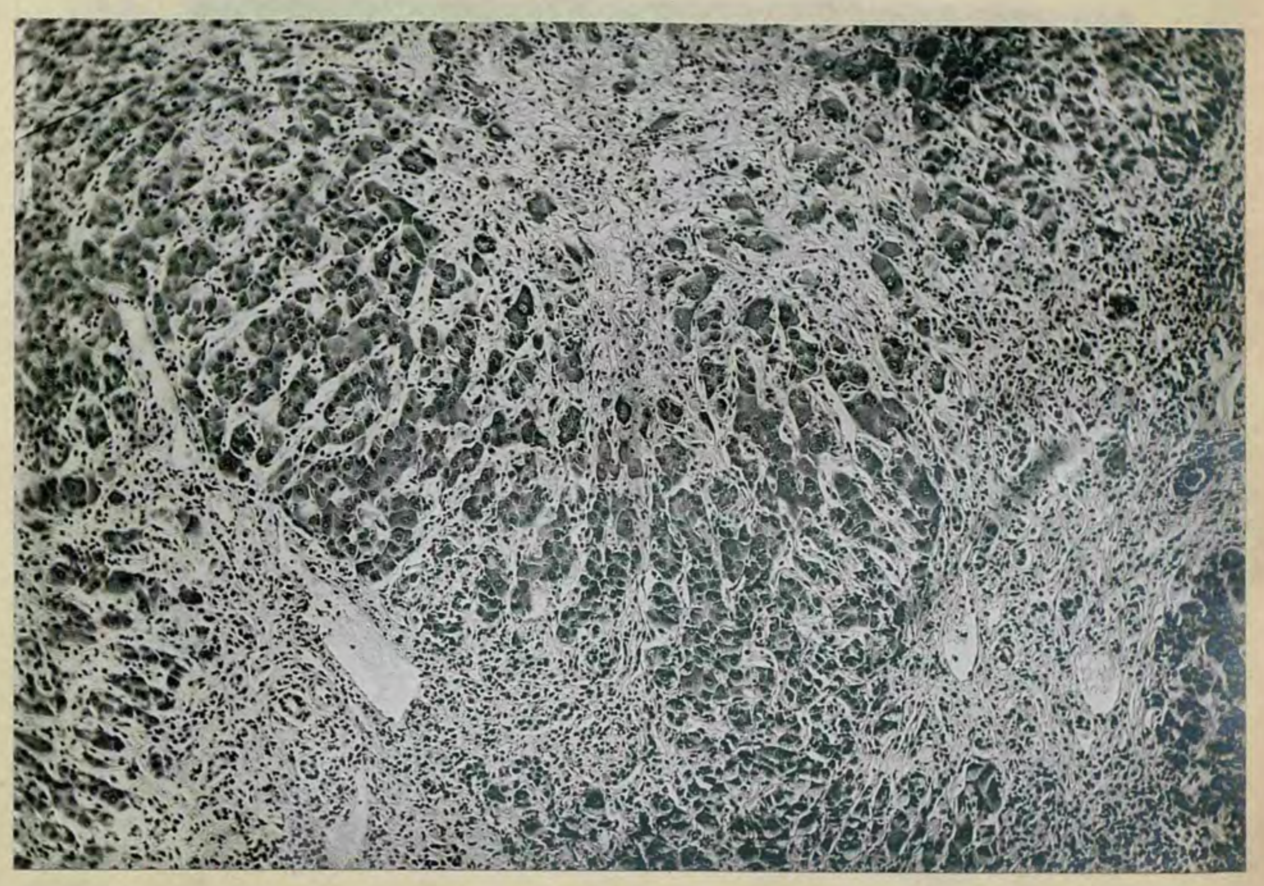


Intravenous cholic acid tolerance.

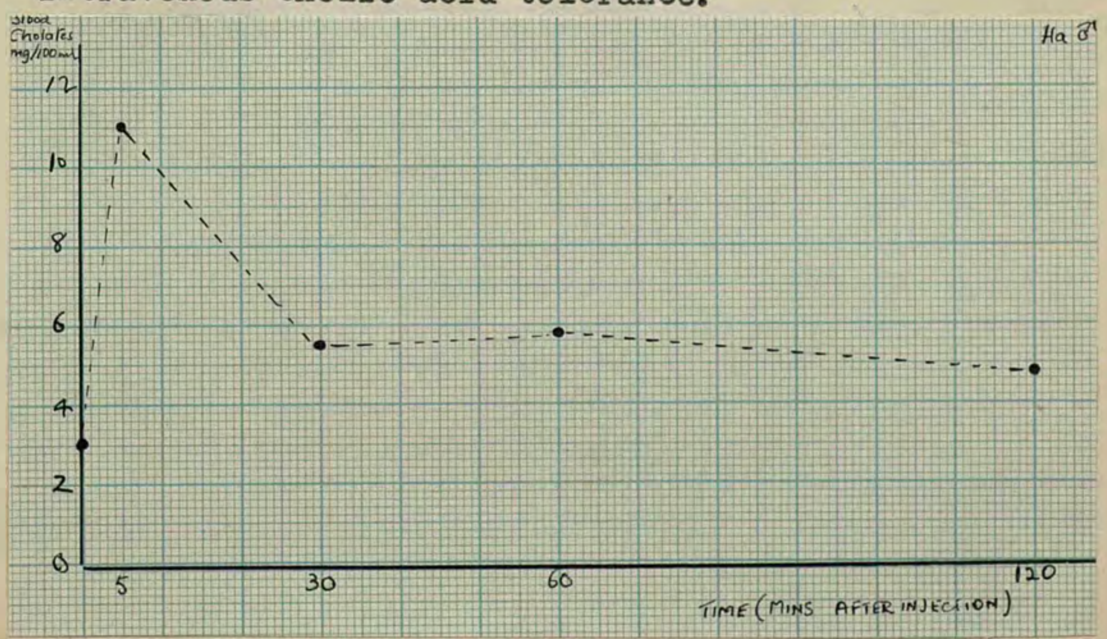


Group D. ACUTE HEPATITIS. Fig. 8.

Grade C.



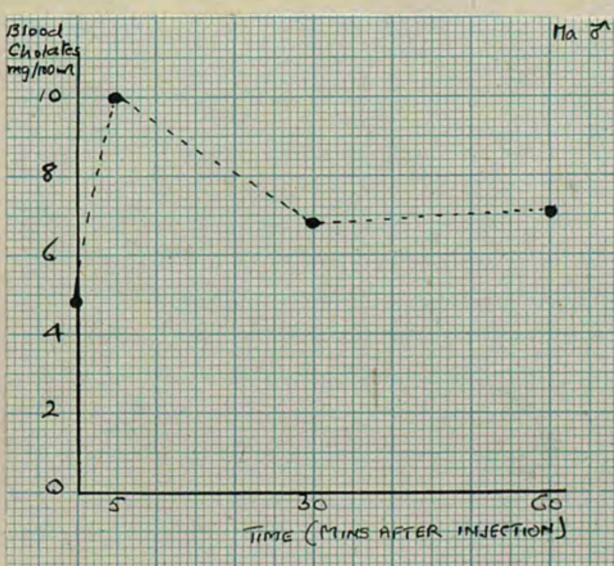
Intravenous cholic acid tolerance.



Group D. ACUTE HEPATITIS. Fig. 9.

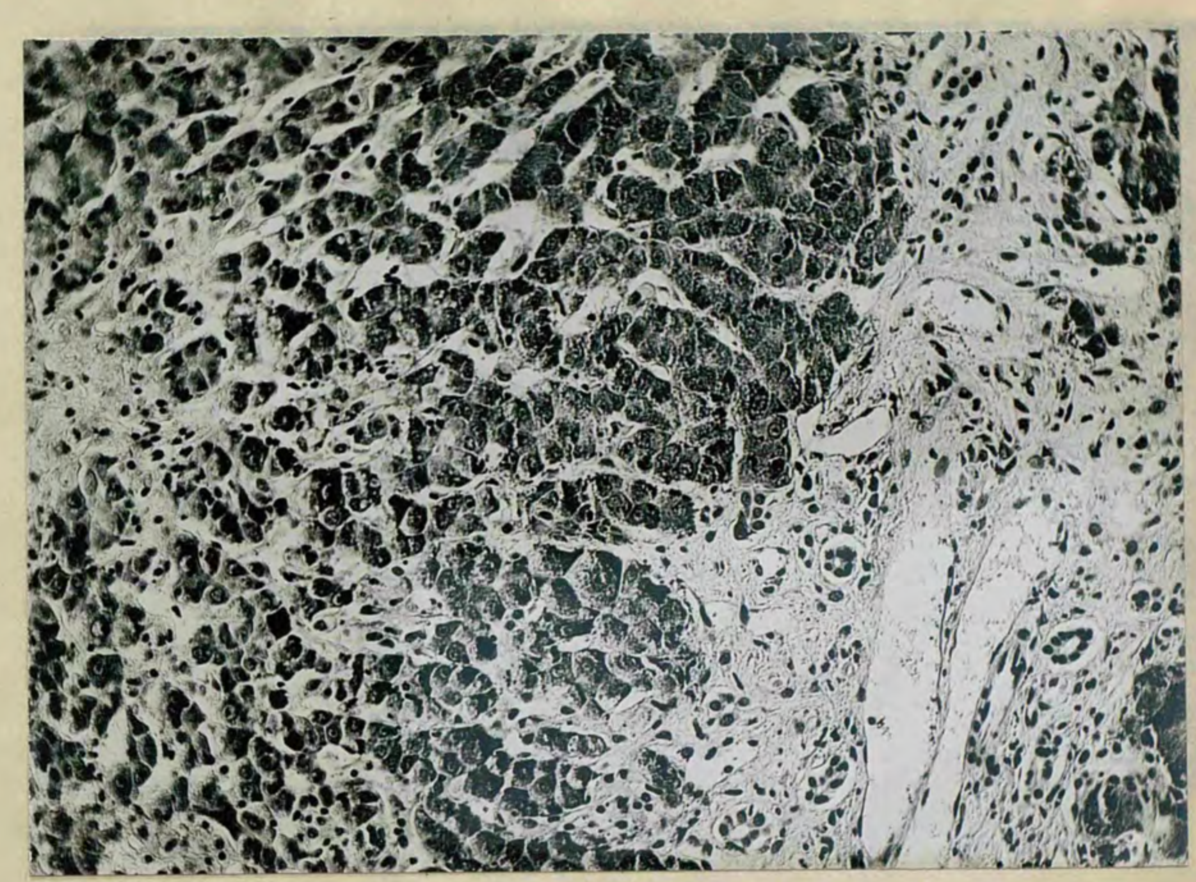
Grade D.

Intravenous cholic acid tolerance.

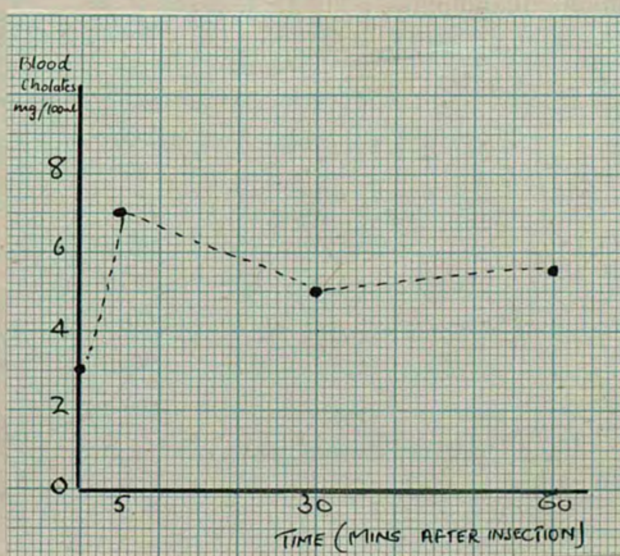


Group D. ACUTE HEPATITIS. Fig. 10.

Liver Biopsy 2.



Intravenous cholic acid tolerance.



Group D. <u>ACUTE HEPATITIS</u>. Fig. 11.

Recovered. Liver biopsy 3.

Intravenous cholic acid tolerance.

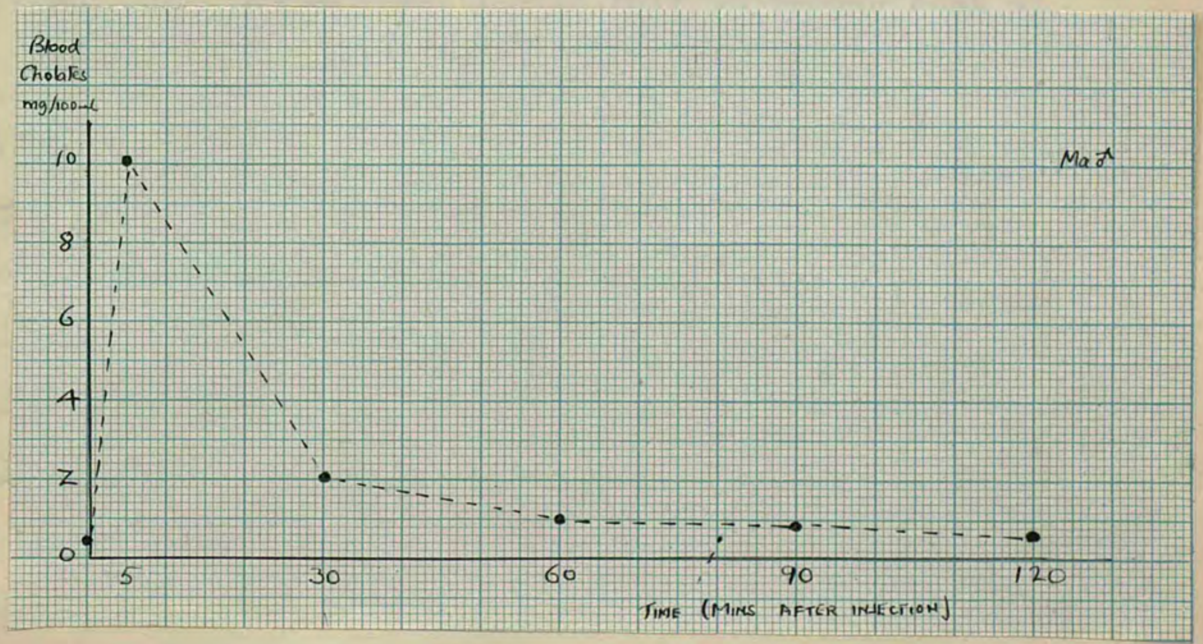


Table 20. Cholic Acid tolerance in Secondary Carcinoma of the liver.

Patient.	Sex	Bilirubin mg/100 ml	Cholic acid tolerance Blood bile acids F	5'	60' mg/100 ml.	Urine bile acids
T.	F.	0.5	1.5	5.0	2.0	- ve
G.	M.	1.2	3.0	8.7	4.0	10. 0
Gy	M.	0.5	2.5	15.0	4.9	- ve
A.	F.	0.8	2.0	7.5	1.5	- ve

E. HAEMOLYTIC JAUNDICE

In one case of the 5 investigated the tolerance was abnormal and in that the original resting level was very low, while the final 60 min. figure is within normal limits. In none of these cases was there any output of bile salts in the urine after injection. Table 21.

Table. 21. The Intravenous Cholic Acid tolerance test in Haemolytic Jaundice

Patient.	Sex	Bilirubin mg/100 ml	F.	5'	60'	Bile acids in urine
L.	M.	2.2	3.0	11.5	2.8	- ve
E.	F.	1.9	3.0	6.0	2.5	- ve
G.	F.		1.0	-	1.2	- ve
F.	M.	3.2	0.8	7.0	2.0	- ve
J.	F.	2.9	1.0	7.5	1.5	- ve

7. Discussion.

Other workers, Table 15, using methods similar to that described here, have obtained comparable results. (Greene, Aldrich & Rowntree,[246] 1927), (Josephson,[237] 1935). Cholates and glyco- and taurocholates, are estimated by this method, but not deoxycholates. It is, however, unlikely that deoxycholic acid occurs in appreciable quantities in normal blood. Cholate production by the liver, is probably steady, apart from a slight decrease at night (Josephson)[196] 1941). Large and unphysiological amounts of bile salts, intorduced into the intestines of experimental animals, produced only a slight rise in blood cholates (Josephson & Rydin,[150] 1936). It is, therefore, not surprising that there is no systemic rise as bile enters the intestine during normal digestion.

The site of formation of cholates has been disputed; after complete hepatectomy in dogs, bile salts were not present in the blood or urine, and injected cholates could be quantitatively recovered from the urine (Bollmann, & Mann,[189] 1936) In cats and rabbits, ligation of the liver vessels produces a fall in blood cholates (Josephson & Rydin,[22] 1938). Both these findings suggest the hepatic formation of bile salts, but Schmidt & Hughes,[168] (1944), failed to confirm this invitro. In experimental animals, hepatic poisons such as chloroform, or carbon tetrachloride inhibit bile salt formation (Smith, & Whipple,[192] (1930), Bollmann & Mann[189] (1936). In hepatitis in

man, the blood cholate level is usually raised. (Josephson,[255] 1939, Turner, Snavely, Grossmann, Buchanan & Foster,[284] 1944) This rise was found in the series of 50 cases of acute hepatitis reported here, but there was no fall with increasing severity of the disease. These different results in man and experimental animals may be associated with differences in hepatic histology. In experimental liver poisoning, the hepatic cells are most affected, the intra-hepatic tree being relatively intact. In infective hepatitis there is hepatic cell necrosis, and also disruption of the liver cell columns, with their associated intercellular bile canaliculi. (Dible, McMichael & Sherlock,[285] 1943). This may cause lessened cholate production and cholate excretion is also obstructed. The highest blood cholate values in this series were found when urobilin was absent from the urine, and the serum bilirubin was rising, which supports this theory. Serial liver biopsies have shown the very rapid recovery which usually occurs in acute hapatitis. The bile channels are quickly restored, and this coincides with the rapid decrease of the blood cholates to normal.

In cirrhosis the blood cholate concentration is very variable. (Josephson,[255] 1939; Rowntree,[246] 1927). This may be associated with the complex histological picture. The number of functioning liver cells is diminished, which decreases cholate production. The vascular tree in the cirrhotic

liver is also diminished (McIndoe,[286] 1928). Excretion of the cholates which are produced, may be impaired. All these factors and probably many others affect the blood cholates level in cirrhosis.
In obstructive jaundice the rise in blood cholates is due to simple retention. Production of bile salts is normal, but their excretion is prevented by biliary obstruction. The level is said to fall in the later stages of the disease, (Rowntree, Greene, & Aldrich,[246] 1927). (Irwin, Johnston & Kopala,[238] 1944). This is usually attributed to diminishing output. In the liver, damage to the hepatic parenchyma increases the longer the obstruction lasts. This fall in blood cholates has not been found in this series. Rising values have been recorded for as long as three months. Probably only very severe damage to the hepatic parenchyma would be reflected in the blood cholate level, and in many cases of obstructive jaundice the damage is not great enough.

It is not surprising that in haemolytic jaundice the blood cholate level is normal, since impairment of liver function is negligible. Similarly, in malignant hepatic metastases, there is usually enough intact liver remaining to maintain a normal cholate concentration in the blood.

Blood cholate estimations might be expected to throw some light on the condition of the liver, since cholates are supposedly only produced by liver tissue, whereas bilirubin

phosphatase and protein can be formed by other tissues as well. The level of these substances do not, it is true, correspond with the blood cholate values, but small degrees of hepatic dysfunction cannot be demonstrated by the bile salt level, which is an insensitive index of the degree of liver damage. The diagnostic application of the estimation is also worthless, as there is considerable overlap both between the values found in cases os obstructive jaundice and hepatitis, and also between cases with liver damage and those without.

The intravenous Cholic acid tolerance test.

Since cholates are probably only synthesised in the liver, the response to an intravenous injection of sodium cholate might reasonably be expected to be of use as a sensitive liver function test. The intravenous Galactose tolerance will only show gross liver damage, hippuric acid synthesis is complicated by renal clearance, and dye retention cannot be used in the presence of jaundice, so it was hoped that intravenous cholic acid tolerance would prove of value in the investigation of liver disease.

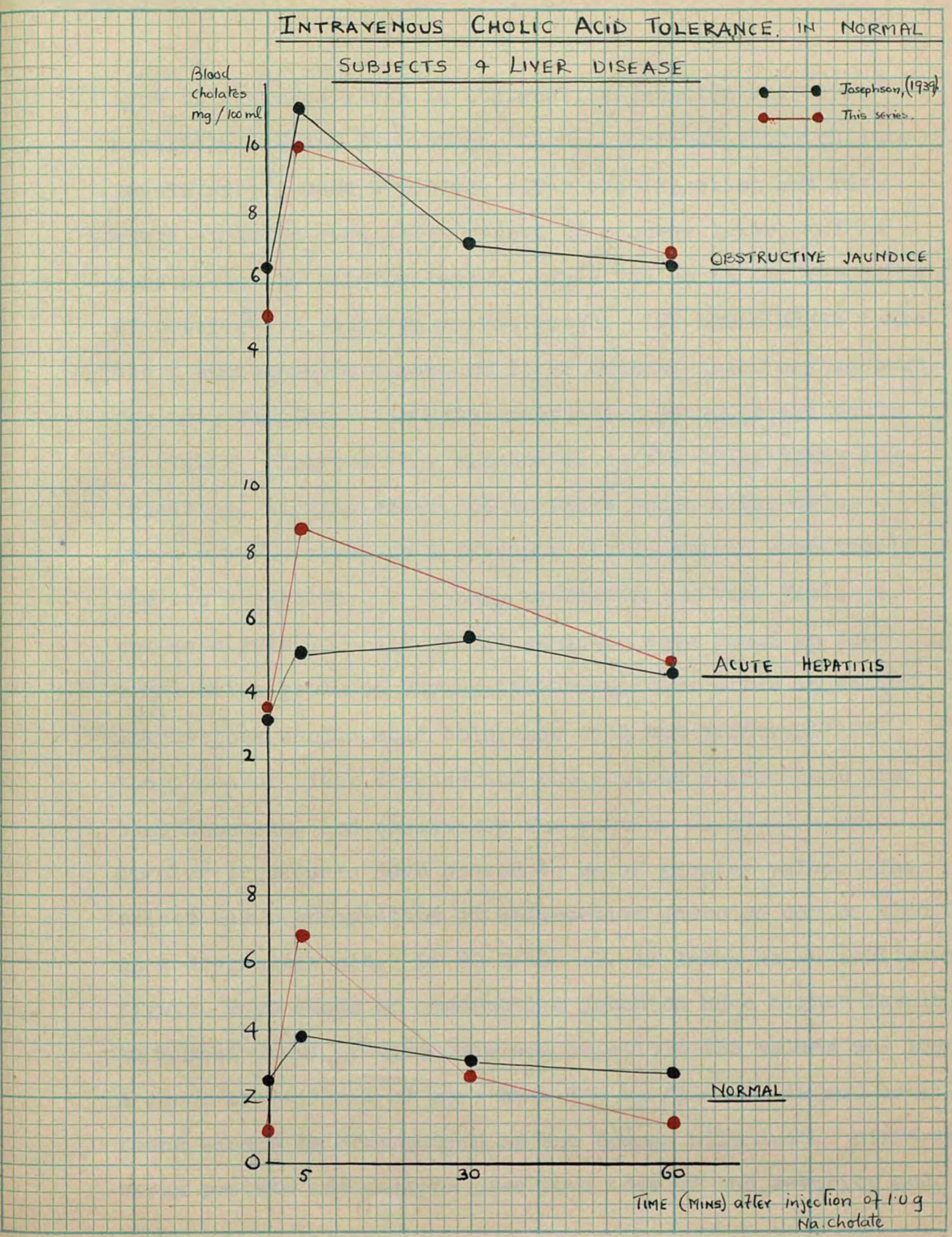
The rapid elimination of injected bile salts in healthy animals and man has been known for many years. (Snell, Green & Rowntree, 1927; Bollmann & Mann,[189] 1936; Chabrol, Cottet & Sallet,[187] 1936; Lichtmann,[191] 1936; Schmidt,[235] 1937). An injection of 250 mg. cholic acid in normal cats and rabbits produced

an increase in the blood cholates, 4 minutes after the injection, of only 6 - 20 mg./100 ml. If the injected cholates had simply been diluted with the animal's blood, the increase would have been about 100 mg/100 ml. Similar results were obtained in man. (Josephson & Larson,[23] 1939; Josephson,[255] 1939) 5 minutes after the injection of 1.0 gm. cholic acid, the blood cholates had risen from 2 or 3 mg/100 ml to 5 or 6 mg/100 ml. If simple dilution had occurred, the corresponding figure would have been 22 mg/100 ml. In half an hour the pre-injection level had been reached. This rapid removal of cholates from the blood could not be entirely due to the liver, since if the liver vessels were ligated, the concentration 5 minutes after the injection was still only 2/3 of what might have been expected, had simple dilution occurred (Josephson, Jungner & Rydin,[22] 1938). As suggested explanation for this was diffusion of the bile salts into the tissues and fixation there. Chabrol, Cottet, & Sallet[187] (1936) found a fixation of this kind with muscle tissue. However, this mechanism cannot be present in jaundice, since in cats with experimental obstructive jaundice there was no disappearance of injected cholates at all, and simple dilution had occurred.

In patients and animals with bile fistulae the liver almost quantitatively excretes bile salts from the blood (Josephson, & Larson,[23] 1939). If, however, bile salts are injected

directly into the portal vein in rabbits, the blood cholates remain high for some time; the liver under these conditions seems unable to excrete the cholates. Josephson, Jungner & Rydin[22] (1938) suggested that such a large local concentration of bile salts might have a paralysing effect on the liver. This effect was not found in cats. In liver disease, hight cholate concentrations are found after a bile salt injection, and the subsequent path of elimination is not altogether clear.
Chabrol, Cottet & Sallet[187] (1936) regarded the liver in jaundiced animals as capable of fixing and storing cholates to an abnormal degree, while any surplus was excreted by the kidneys. Jungner, Rydin & Josephson[22] (1938) claimed that in obstructive jaundice bile salts are absorbed by the liver, and then enter the hepato-bilio-lymphatic circulation, thus disappearing quickly from the blood as the liver cells are damaged and cannot absorb them, and the vessel walls are already loaded with bile salts.
The elimination of injected cholates was, therefore, used as a liver function test by Josephson, Jungner & Rydin[150] (1936). In animals with experimental jaundice of different kinds they found rapid disappearance of the injected cholates in obstructive jaundice, and high blood levels for some time after the injection in hepatitis. In man, Josephson[255] (1939) found the same differentiation between the two kinds of liver disease. In this series, there was no difference in the

Graph 17



type of response given in obstructive jaundice and acute hepatitis. (Graph 17.) High, though variable blood cholate levels were found 5 minutes after the injection and the blood cholates were still raised 60 minutes later, and in some cases 120 minutes later. This was so in all the cases of obstructive jaundice investigated. In these cases there was a considerable amount of liver damage, which may account for this finding. In both obstructive jaundice and acute hepatitis, cholates were eliminated in the urine after the injection, in many cases, up to three hours afterwards. There was, however, no difference in the amount excreted in the two conditions. Urinary excretion was very variable, but as a rule 50 - 100 mg. of cholates were found in the total urine produced during the three hours after injection. Intravenous cholic acid tolerance will not differentiate the types of liver disease and the cholate estimations are time-consuming and unsuitable for routine clinical work. Simpler procedures have proved to be of greater value. The raised serum phosphatase in obstructive jaundice can be used as a diagnostic aid. If 30 U/100 ml. is taken as an arbitrary figure, most cases of obstructive jaundice have higher levels, and in most cases of cirrhosis and hepatitis the values are lower. A serum cholesterol of more than 300 mg./100 ml. is not usually found except in obstruction of the common bile duct. Differential serum protein estimations give some idea of the severity and

and prognosis of acute and chronic parenchymatous liver disease. Total serum protein estimations are of little practical use.

In conclusion, it appears that as much practical diagnostic information can be obtained from a single venous blood sample, used for the estimation of the serum bilirubin and the serum alkaline phosphatase, as from any of the more complicated liver function tests.

SUMMARY.

1. The mean blood cholate level in 50 normal subjects was 1.6 mg./100 ml.
2. There was little diurnal variation in the blood cholate level. During a period of several weeks the level fluctuated within normal limits.
3. In 17 cases of obstructive jaundice, the mean blood cholate level was 3.7 mg./100 ml. The highest values were found in this group. When the obstruction is relieved by operation, a considerable fall occurs in the blood cholate level.
4. In 18 cases of cirrhosis, the mean blood cholate level was 1.8 mg./100 ml.
5. In 50 cases of acute hepatitis, the mean blood cholate level was 2.8 mg./100 ml. There is considerable overlap between the values found in this group and in obstructive jaundice.
6. Cholic acid tolerance, intravenous Galactose tolerance, and Hippuric acid synthesis are all impaired in the more severe grades of hepatitis, and in obstructive jaundice. No constant biochemical abnormalities are found in latent cirrhosis, but in active cirrhosis, liver dysfunction is shown by all the methods used. Serum bilirubin and serum alkaline phosphatase estimations are of the most diagnostic value.
7. In normal subjects, an intravenous injection of 1.0 g. sodium cholate, raises the blood cholate level to 5-6 mg./100 ml. 5

mins. after the injection. The bloodstream is cleared of cholates in 45 mins. No cholates appeared in the urine after the injection.

8. In 16 cases of obstructive jaundice the blood cholate level at 60 mins. was 2-3 mg. higher than the initial level. In 5 cases a blood sample was taken 2 hours after the injection, and in each of these the value was still raised. Bile salts appeared in the urine in 13 out of 16 cases.

9. The bile acid level 60 mins. after the injection, was not significantly raised in 5 out of the 13 cases of cirrhosis. Bile acids appeared in the urine in only 2 cases. Urobilinuria was present in every case.

10. There was no difference in the type of curve obtained in response to the injection, in obstructive jaundice and acute hepatitis. Urobilinuria is absent in the more severe grades of hepatitis, and in these cases, cholates appeared in the urine after the injection.

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British Postgraduate Medical School. May, 1947.

The Estimation of Small Amounts of Glycogen in Tissue. By V. M. Walshe

The tissue is placed in a weighed tube containing 2 ml. 30 % KOH (Good, Kramer & Somogyi, 1933). The tube is weighed again, and warmed on a water-bath until the tissue dissolves. 2 ml. 95 % ethanol are added, and the tube immersed in boiling water until the ethanol boils. The precipitate is centrifuged hard for 10 min. and well drained. 2 ml. 50 % ethanol are added and the precipitate dispersed with shaking and tapping. Centrifugation and washing with 50 % ethanol are repeated until the supernatant is no longer alkaline to phenolphthalein (Davies & Francis, 1941). The last traces of ethanol are removed by warming and the precipitate is dissolved in 2 ml. 0·6 N-perchloric acid, and hydrolyzed for $2\frac{1}{2}$ hr. on a water-bath.

All glass apparatus should be used such as centrifuge tubes with ground-glass stoppers to which air condensers are fused. ($7 \times \frac{1}{2}$ in.) soft glass test-tubes are satisfactory, if, after the perchloric acid has been added, they are drawn out in a flame to make an air condenser.

The perchloric acid is neutralized with 2 ml. 0·6 N-KOH (accurately standardized against the acid used for the hydrolysis), and the white crystalline precipitate of potassium perchlorate spun down. Samples of the supernatant are used for glucose estimations, by the method of Haslewood & Strookman (1939).

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(b) The inhibition by Cu^{++} plus ascorbic acid is of the same magnitude as with Cu^{+} alone. The inhibition produced by Cu^{+} is not increased by the addition of ascorbic acid.

(c) Other reducing substances containing a dienol group and capable of reducing $Cu^{++} \rightarrow Cu^{+}$, e.g. hydroxytetronic, reductic and dihydroxymaleic acids, resemble ascorbic acid in their power to inactivate urease in the presence of Cu.

(d) Urease inactivated either by Cu^{++} plus ascorbic acid or by Cu^{+} alone may be reactivated by H_2S, or by cysteine.

Dehydroascorbic acid does not inhibit urease, even in the presence of Cu. 2, 3-Diketogulonic acid (produced by mutarotation when solutions of dehydroascorbic acid are left standing) inhibits only in the presence of Cu, but to a less extent than does ascorbic acid.

Of the metallic salts tested, Cu^{++} and Au^{+++} alone had their toxicity increased by the addition of ascorbic acid. With Hg^{++} salts the toxicity was reduced by ascorbic acid.

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Relation between Glycolysis and Tissue Integrity in Gastric Mucosa. By C. LUTWAK-MANN and A. M. BARRETT

The glycolysis of the gastric mucosa (rat) was studied manometrically and chemically using complete transverse sections (10–15 mg. dry weight) of the stomach usually in the region of the cardiac glands. This technique was adopted because if the mucosa was separated from the rest of the wall it rapidly disintegrated, with loss of protein into surrounding saline and a fall in enzymic activity; stomach wall devoid of mucosa was

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THE POST-HEPATITIS SYNDROME

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Caravati (1944) has described cases of persistent disability following postvaccinial (yellow fever) hepatitis and has designated the condition the "post-hepatitis syndrome."

Benjamin and Hoyt (1945) report a similar series, and during the past two years we have studied a group of soldiers in whom symptoms and usually an enlarged liver have remained after clinical recovery from acute hepatitis. Besides studying the clinical features and making biochemical investigations, we have used the aspiration technique of liver biopsy to study hepatic histology. At the present time, when so many men who have had hepatitis are returning to civilian life, it seems important to report the findings.

Our 20 patients were soldiers of the British, Canadian, and Czechoslovak Armies. All were grade A before contracting acute hepatitis. In 18 the hepatitis was of the simple "infective" type; in 2 it had followed arsenotherapy for syphilis.

The laboratory methods used were the estimation of serum-bilirubin, cholesterol, alkaline phosphatase (King and Armstrong 1934), and total and differential serum proteins. The colloidal-gold reaction (Maclagan 1944), the bromsulphthalein test with a 5 mg./kg. dose and taking 5-min. and 30-min. samples (Helm and Machella 1942), and the intravenous hippuric-acid test (Sherlock 1946a) were also used. Routine urine examination included urobilinogen by Ehrlich's aldehyde reagent, and bilirubin by Hunter's test (Pollock 1945).

* In receipt of a maintenance grant from the Medical Research Council, who have also defrayed the expenses of this investigation.

Aspiration liver biopsy was performed by the method previously described (Sherlock 1945).

FEATURES OF THE DISORDER

The presenting features were as follows :

	No. of cases		*No. of cases*
Fatigue	18	Fat-tolerance ..	6
Weight-loss	11	Relapse of hepatitis..	8
Anorexia	12	Palpable liver ..	16
Abdominal discomfort	10	Palpable spleen ..	3

Preceding Hepatitis and Relapses.—Of the 20 patients 6 had had more than one acute attack of hepatitis ; 1 patient was said to have had six. The last acute episode took place 2–24 months previously.

Symptoms.—The usual complaint was lack of energy and exhaustion on minimal exertion. Inability to regain the weight lost during the acute attack was common. Gastro-intestinal symptoms were prominent ; some patients had a poor and variable appetite, with sometimes an aversion to fatty foods. The men were very faddy about their diet. Right upper abdominal discomfort, often aggravated by exertion, was occasionally present.

Consumption of Alcohol.—Of the 20 patients 10 confessed to excessive alcohol intake, 6 took moderate amounts, and 4 were almost teetotal.

Mental State.—A detailed psychiatric examination was not attempted. The British patients were on the whole psychologically ill-balanced. They were extremely introspective and unduly apprehensive about their livers. One was an Army deserter, another had just been invalided from the Services with " effort syndrome." The Canadian group showed better understanding of their symptoms. All, however, had been warded together, and a similarity in the wording of their case-histories was often noticed. Moreover, they were in hospital at the end of European hostilities, when there was delay in repatriation to Canada, and it was believed that sick men would receive priority.

General Examination.—Despite the complaint of weight-loss, the general development of the group was excellent. Spider angiomata were not seen.

Hepatomegaly.—The most common positive finding was hepatomegaly. The liver edge, smooth and rubbery

in consistence, could be felt on inspiration 2–7 cm. below the right costal margin in the nipple line. Tenderness was not present. Liver tenderness on fist percussion over the right lower ribs (Barker et al. 1945) was not elicited.

Splenomegaly.—In 3 patients the spleen could just be palpated under the left costal margin.

Urine Analysis.—This was usually normal, but 5 patients showed a trace of urobilinogen in an early morning specimen of urine. Hunter's test for bilirubin was consistently negative.

Biochemical Investigations.—In every patient the serum-bilirubin, the total and differential serum proteins, and the bromsulphthalein test were normal. The serum-cholesterol level was high in 7 cases ; in 3 of these it was greater than 300 mg. per 100 ml. Slight changes among the other estimations were a serum-phosphatase of 14 units per 100 ml. in one patient, a weakly positive colloidal-gold reaction in two patients, and in a further two cases the excretion of hippuric acid was at the lower limit of normal (0·7 g. as sodium benzoate). The biochemical observations on the whole, therefore, gave essentially normal results. Caravati (1944) found a low fasting blood-sugar level and flat oral glucose-tolerance curves in some of his patients. Glucose-tolerance tests were performed in 5 of our subjects and gave normal results.

Hepatic Histology.—There was no evidence of continuing hepatitis or of cirrhosis. The lobular pattern was not disturbed. The hepatic cells were usually normal and contained their normal complement of glycogen. In 2 instances the glycogen was slightly deficient, and in another there was patchiness of glycogen. Iron was absent both from the Kupffer and the hepatic cells. In one patient who had previously had malaria, there was iron in both situations. In ten sections some excess of fat was seen in the liver-cells. It usually took the form of scattered fine droplets evenly distributed through the lobules. In another case the fat was peripheral. Slight fatty change was the only abnormality encountered with any frequency. The Kupffer cells were normal. In 3 patients, all within three months of the initial attack of hepatitis, excess of fibrous tissue was seen in the portal tracts. The picture here resembled residual portal scarring following hepatitis (Dible et al. 1943).

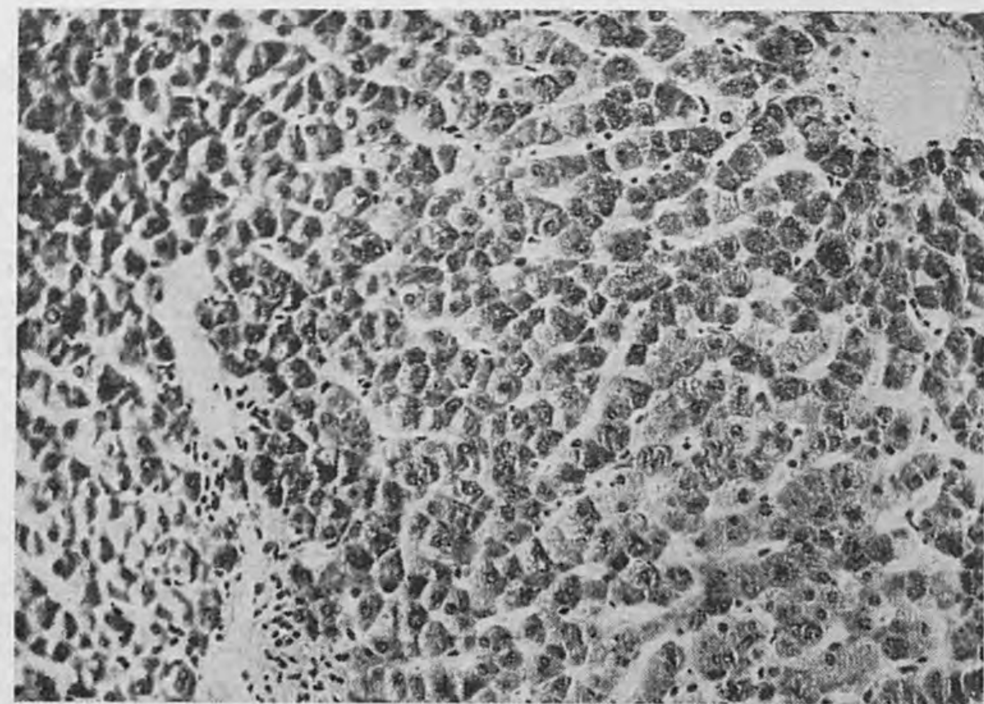

Fig. 1—Normal hepatic structure. Case 1. Best's carmine stain. (× 120.)

ILLUSTRATIVE CASE-RECORDS

CASE 1.—A British officer, aged 31, was fit until October, 1943, when he had infective hepatitis in Libya. He was jaundiced three months and lost two stone in weight. He was invalided home in April, 1944. Since the hepatitis he had had persistent right upper abdominal discomfort, made worse by exercise. Fatty foods caused nausea and flatulence. Appetite was variable. There was exhaustion on walking only half a mile, and some dyspnœa on exertion. In October, 1944, he was again slightly jaundiced and in bed a week. He was a moderate drinker of alcohol.

On examination (March 10, 1945) he was a tall well-developed man, not jaundiced. The smooth rounded liver edge could be palpated 6 cm. below the right costal margin. Tenderness was not present. The spleen was not felt. Urine analysis was normal. The biochemical investigations were normal. Aspiration liver-biopsy sections showed normal liver histology (fig. 1).

This patient was extremely introspective and worried about his health. He had had advice from many doctors, both Army and private, before the present investigation. Even when he was told that his liver was normal the symptoms persisted.

CASE 2.—A Canadian N.C.O., aged 31, had had infective hepatitis at 16 years of age, when he was jaundiced a month. In September, 1944, in Italy, while having arsenotherapy

for syphilis, he again became jaundiced for three weeks. The symptoms were those of acute hepatitis.

Since then he had complained of lack of energy and dyspnœa on exertion. The appetite was poor and there was much heartburn and gastric flatulence. During the jaundice the patient lost a stone in weight ; this had not been regained. In May, 1945, there was a further attack of hepatitis ; jaundice lasted about a week. The symptoms persisted. Cholecystograms were normal. Patient drank a lot of beer, usually six pints a night, with extra beer and spirits at the week-end ; this had continued to the date of this investigation (July, 1945).

On examination he looked well. He was not underweight. The liver was palpable 4 cm. below the right costal margin. The spleen was not felt. Urine analysis was normal. The only abnormal biochemical finding was a serum-cholesterol level of 309 mg./100 ml.

Aspiration liver-biopsy sections showed a slight excess of fat within the hepatic cells at the periphery of the lobules ; the portal tracts contained a little excess fibrous tissue and showed some round-celled infiltration (fig. 2).

The repeated attacks of jaundice had made both the patient and his medical advisers suspect permanent liver

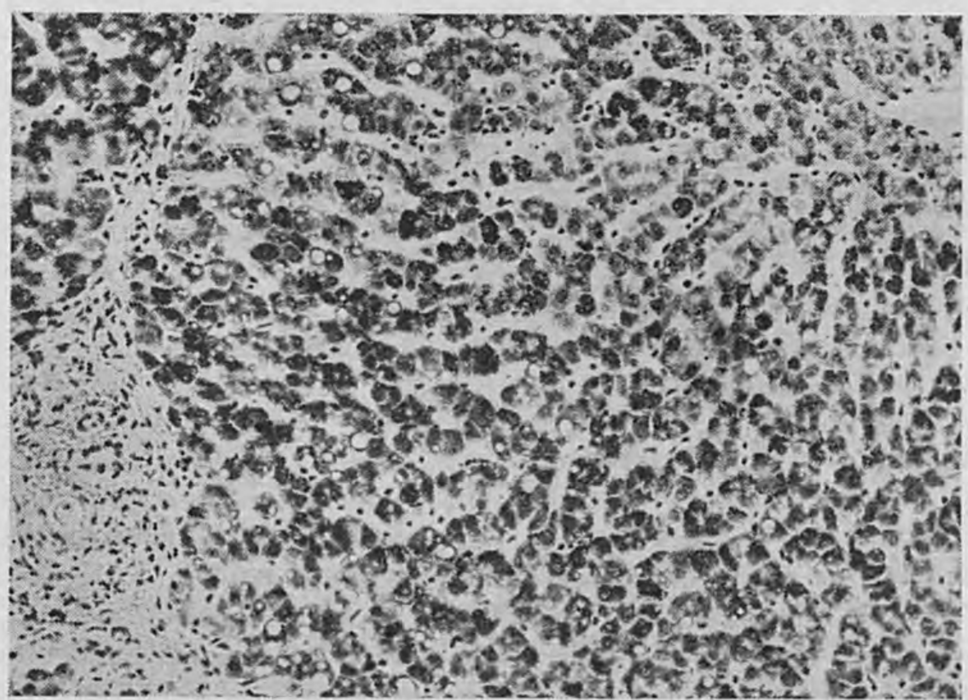

Fig. 2—To left of the figure a portal tract shows increased fibrous tissue and is infiltrated with mononuclear cells ; at the periphery of the lobule the hepatic cells show slight fatty change. Case 2. Best's carmine stain. (× 120.)

damage. When the present investigation showed this not to be the case he was much relieved and became symptom-free.

DISCUSSION

In the group studied there is no causal relationship between the slight biochemical and the hepatic histological changes and the symptoms. Similar findings have been observed in patients now symptom-free but within six months of clinical recovery from acute hepatitis. In 10 of 15 such subjects the liver was still palpable. Hepatic sections usually showed portal-tract scars, and in 7 excess fat was present in the liver-cells. A raised serum-cholesterol level is also sometimes found during recovery from hepatitis (Sherlock 1946b). Benjamin and Hoyt (1945) believe that the symptoms may have a psychotic basis ; the psychoneurotic patterns observed in our patients were very similar to those recorded by these authors. Acute hepatitis is nearly always an unpleasant experience. It usually lasts a minimum of 3–4 weeks. Convalescence is slow. If the illness relapses, as it did in many of this group, fear may arise of further attacks and of permanent liver damage. This is accentuated if a number of men are warded together and repeatedly examined with a view to determining liver size. The condition has not been seen in civilian patients. It is commoner in those serving overseas. Some men feel the disease may provide an opportunity for repatriation. In the type of person affected the condition is somewhat analogous to " effort syndrome," with symptoms focused on the liver and gastro-intestinal tract rather than the heart.

The hepatomegaly may in some instances be related to the histological picture of fatty change and residual portal-tract scarring. A more likely cause is the downward displacement of a normal-sized liver by the diaphragm. Some patients, with practice, become very efficient at " pushing down the liver." On inspiration the lower liver edge has been observed to move down 6 cm. in one of these patients ; an impalpable liver is thus easily felt. Similar considerations apply to the spleen. Moreover, a palpable liver, usually but not constantly due to downward displacement, is not uncommon in normal subjects. On ten occasions such a liver has been subjected to aspiration biopsy with entirely normal results.

The importance of the syndrome is in its distinction from the serious organic sequelæ known rarely to occur after hepatitis (Krarup and Roholm 1941, Dible et al. 1943, Rennie 1945). We have studied 6 patients in whom cirrhosis could be related to a preceding acute hepatitis: 1 showed hepatomegaly, splenomegaly, and abnormal results for all the biochemical methods used; 2 were symptom-free and presented only hepatomegaly; the remaining 3 had clinical features and biochemical findings identical with the series now reported. Clinical and laboratory findings cannot constantly distinguish organic from possibly psychogenic sequelæ; but a definite conclusion can usually be reached after study of aspiration liver-biopsy sections. The importance of this method is emphasised. All the patients volunteered for this procedure and usually derived great benefit from the reassurance possible when results were known.

This sequel of hepatitis may be prevented if patients with the same condition are not herded together. Patients apparently recovering normally should not be examined too often. The condition is unlikely to occur with any frequency in civilians. Treatment consists in reassurance after the fullest possible investigation.

SUMMARY

In 20 patients fatigue and gastro-intestinal symptoms arose, usually with hepatomegaly, after acute hepatitis.

Serum-bilirubin, phosphatase, and differential protein estimations, the colloidal-gold reaction, the intravenous hippuric-acid test, and the bromsulphthalein test showed no abnormalities. There was an occasional rise in serum-cholesterol level.

Hepatic sections obtained by aspiration biopsy were usually normal. In some sections slight fatty change in the liver-cells and occasional scarring in the portal tracts could be seen.

No difference was found between these results and those obtained in subjects who had recovered from acute hepatitis and were now symptom-free.

The possible psychogenic basis of the symptoms is discussed.

The palpable liver seems due to downward displacement of the liver edge rather than to enlargement.

The value of aspiration liver biopsy in the diagnosis of this syndrome from post-hepatitis cirrhosis is emphasised.

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THE USE OF A PORTAL ANASTOMOTIC VEIN FOR ABSORPTION STUDIES IN MAN.

By SHEILA SHERLOCK and VERYAN WALSHE.

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THE USE OF A PORTAL ANASTOMOTIC VEIN FOR ABSORPTION STUDIES IN MAN.

By SHEILA SHERLOCK (Beit Memorial Research Fellow) and
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A PATIENT suffering from hepatic cirrhosis was seen in whom a large anterior abdominal wall vein was believed to communicate with the portal venous system. Observations were made on the contents of this vein during intestinal absorption. Although the studies were brought to a premature conclusion by thrombosis of the vein, the results are reported in the hope that they may stimulate interest in the possible research value of such veins in similar patients.

CASE REPORT.

F.M., an old age pensioner aged 71, was admitted to hospital on 8.11.45.

History : For several years there had been recurrent epistaxes ; now occurring nearly every week and initiated by blowing the nose. Appetite was fair and most foods were well tolerated. The bowels were constipated and the motions occasionally streaked with blood. The early morning urine was sometimes rather dark in colour. There was morning cough with white sputum. The patient thought he was losing weight, the exact amount being uncertain. Past health had been good and he had never suffered from jaundice. He drank 1 pint of beer daily, and lived alone in a single room, his diet being mainly pies and breakfast sausage. He did not eat his meat, bacon or butter rations and rarely took vegetables.

Examination showed a thin old man. "Spider" angiomata suggestive of liver disease were seen on the cheeks. Dilated vessels on the nasal septum were a possible source of the epistaxis. The chest showed a few dry rhonchi but no moist sounds. The abdomen was not distended and shifting dullness could not be elicited. A large dilated, tortuous superficial abdominal vein in the right lower quadrant (Fig. 1) was non-pulsatile and there was no murmur over it. On emptying it rapidly filled from above downwards. Superiorly the vein emerged about 3 cm. above the umbilicus, apparently through a paraumbilical hernia. Inferiorly it joined the great saphenous vein just below the inguinal ligament. The liver edge was firm and easily palpable 4 cm. below the right costal margin in the nipple line. The spleen could not be felt. There was no peripheral œdema. Rectal examination showed internal hæmorrhoids. *Urine* : S.G.1015, contained no albumin or bile pigments. Urobilinogen was present in an early morning specimen.

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* Working for the Medical Research Council.

We are indebted to Dr. Duncan White and the staff of the Radiology Department for their co-operation ; to Professor E. J. King, in whose department many of the biochemical estimations were made ; to Mr. E. V. Willmott for the photography ; and especially to Major Kendal Dixon and Lt.-Col. W. R. M. Drew for assistance with the fat tolerance tests.

Investigations : Serum bilirubin 0·9 mg. per 100 c.c. ; serum alkaline phosphatase 15 units per 100 c.c. ; serum cholesterol 265 mg. per 100 c.c., serum proteins 6·2 g. per 100 c.c. with 3·5 g. albumin, 2·7 g. globulin, and A/G ratio 1·3. Serum colloidal gold reaction (18) negative. Oral hippuric acid synthesis test (20), 2·5 g. (as sodium benzoate) excreted in 4 hours. *Blood count* : Hb. 10·2 g. per 100 c.c. (65% Haden normal), R.B.C. 4,600,000 per c.mm., W.B.C. 7,000 per c.mm.. Wassermann reaction negative. *Barium meal* examination showed no gastrointestinal abnormality.

Aspiration liver biopsy (21) : sections show a hepatic cirrhosis. Surviving liver cells, apart from some fatty change at the periphery of the nodules, contain their usual complement of glycogen and appear healthy.

As this anastomotic vein probably communicated with the portal venous system, it was considered that its blood might be draining the alimentary tract. The abdominal vein was thin-walled and tortuous which made sampling difficult. This difficulty and the consequent discomfort to the patient limited the frequency of sampling. Arm veins also were small and difficult to needle.

Preliminary metabolic considerations.

Weight of patient 60 kg.. Height 148 cm..

During the period of study the patient consumed a weighed diet of 2,175 calories daily ; 359 g. carbohydrate, 133 g. protein and 23 g. fat. 30 g. brewer's yeast daily was included.

Over two three-day periods on this regime the daily output of urinary nitrogen averaged 9·3 g., the fæcal nitrogen 1·5 g., and the fæcal fat 7·2 g.. The patient thus showed a positive nitrogen balance, and on this diet the fæcal fat was within normal limits. During his stay in hospital he gained 8 kg. in weight. All the observations were made in the morning, the last meal being at 8 p.m. the previous night.

Carbohydrate tolerence tests.

Galactose. 40 g. galactose in 50 per cent. solution was given orally and one hour later samples were taken from the abdominal wall vein and a right antecubital vein.

The galactose content of the abdominal vein was 20 mg. per 100 c.c., and that of the arm vein 7 mg., per 100 c.c..

Lævulose and Glucose. To facilitate more frequent sampling drip infusions of heparinized normal saline were set up in both the abdominal and an antecubital vein. Fasting venous samples were removed from both sites, and then 50 g. lævulose and 50 g. glucose were given by mouth. Further venous samples were taken 30, 60 and 90 minutes later.

From Table I it is seen that :—

1. In the fasting state the glucose concentrations in the two veins is the same. No lævulose can be detected in either. In later experiments a fasting sample was not taken from the abdominal vein : its composition was assumed to be identical with that of the systemic.

2. During the 90 minutes after the sugar was given, the glucose and lævulose content of the abdominal vein is conspicuously greater than that of the antecubital vein.

3. The highest value for glucose in the abdominal vein occurred at 30 minutes, that for lævulose at 60 minutes.

4. During the absorption period, in neither vein was there any significant change in the serum potassium or serum ester phosphate concentrations.

TABLE I.

Biochemical changes in the abdominal wall vein and the antecubital vein after oral glucose and lævulose.

Time after sugar minutes	Concentrations in abdominal wall vein				Concentrations in antecubital vein			
	Glucose mg/ 100 c.c.	Lævulose mg/ 100 c.c.	Ester Phosphate (as P.) mg/ 100 c.c.	Potassium mg/ 100 c.c.	Glucose mg/ 100 c.c.	Lævulose mg/ 100 c.c.	Ester Phosphate (as P.) mg/ 100 c.c.	Potassium mg/ 100 c.c.
0	112	0	0·6	21	112	0	0·6	19
30	230	25	0·5	20	182	8·4	0·5	—
60	123	84	0·7	22	84	24·6	0·5	20
90	159	24	0·7	21	132	6·6	0·2	21

Fat tolerance tests.

These were done by the method of Dixon, Drew and Samuel (8). The meal consisted of 75 g. dairy butter prepared with a barium sulphate suspension. After a fasting blood sample had been taken from an antecubital vein, a duodenal tube with mercury tip was passed into the second part of the duodenum and its position confirmed by X-ray screening. The meal was given down the tube and seen to pass through the duodenum and enter the upper coils of jejunum. Venous samples were taken from the abdominal and antecubital veins two and three hours after the meal.

From Table II it is seen that no significant change in serum opalascence, lipid phosphorus, cholesterol, total fatty acid, non-phospholipid fatty acid or neutral fat concentrations could be demonstrated. Results for a typical normal control subject are also shown.

This result was most unexpected and the observation was therefore repeated. This time 50 g. glucose was included in the meal, and its rate of progress was followed radiologically at intervals for 2 hours. Although the rate of transit of the meal through the intestines was normal, and although a glucose concentration increase was shown in both veins, that in the

TABLE II.

Biochemical changes in the abdominal wall and antecubital veins after a fatty meal with and without added lipase.

	Abdominal wall vein						Antecubital vein					
Minutes after fatty meal	Lipid phosphorus mg/100 c.c.	Cholesterol mg/100 c.c.	Total fatty Acid M.E.Q./ 1,000 c.c.	Non-phospholipid Acid M.E.Q./ 1,000 c.c.	Neutral fat mg/100 c.c.	Serum opalescence	Lipid phosphours mg/100 c.c.	Cholesterol mg/100 c.c.	Total fatty Acid M.E.Q./ 1,000 c.c.	Non-phospholipid fatty acid M.E.Q./100 c.c.	Neutral fat mg/100 c.c.	Serum opalescence
Fatty meal alone.												
0	—	—	—	—	—	—	7·7	225	12·8	8·7	260	0
120	7·9	190	11·8	7·6	230	0	7·7	171	11·8	7·6	230	0
180	8·2	212	12·1	7·7	230	0	8·0	183	12·1	7·9	240	0
Fatty meal + lipase.												
0	—	—	—	—	—	—	4·8	101	12·6	8·6	250	0
60	5·4	102	12·6	8·1	240	0	5·3	104	12·2	7·8	230	0
120	5·3	96	13·3	8·9	270	+	5·4	95	12·2	7·8	230	0
Normal control subject.												
0	—	—	—	—	—	—	8·5	209	8·5	4·5	130	0
120	—	—	—	—	—	—	10·6	224	10·6	6·2	180	+
180	—	—	—	—	—	—	14·9	211	14·9	10·4	310	++

TABLE III.

Biochemical changes in the abdominal wall and antecubital veins after a protein and carbohydrate meal.

	Concentrations in abdominal wall vein			Concentrations in antecubital vein		
Minutes after meal	Glucose mg/100 c.c.	Non-protein Nitrogen mg/100 c.c.	Urea Nitrogen mg/100 c.c.	Glucose mg/100 c.c.	Non-protein Nitrogen mg/100 c.c.	Urea Nitrogen mg/100 c.c.
0	—	—	—	94	31	14
120	119	66	15	72	46	15

abdominal vein being greater than that in the antecubital, there was still no change in the fatty substances. It was decided to repeat the experiment a third time and to give a lipase preparation with the butter fat.

The lipase was made from fresh pig's pancreas (5) and its potency tested. 100 c.c. of this extract was mixed with the fat meal and given under X-ray control into the second part of the duodenum. Venous samples were taken from an arm vein before the meal and from both arm and abdominal veins 1 and 2 hours afterwards.

From Table II it is seen that in the abdominal vein 2 hours after the fatty meal there is a slight serum opalescence and an increase in total fatty acid, non-phospholipid fatty acid and neutral fat concentrations. The concentrations of phospholipid and cholesterol show no change. During the 2-hour period there has been no detectable systemic lipæmia.

Protein tolerance test.

A fasting sample was withdrawn from an antecubital vein and the patient then ate a meal consisting of meat, cabbage, potatoes, bread and concentrated skimmed milk. It was equivalent to 86 g. carbohydrate, 62 g. protein and 6 g. fat. Two hours later samples were withdrawn from the abdominal vein and from the antecubital vein.

From Table III it is seen that (1) 2 hours after the meal there is a significant difference between the glucose concentrations of the two veins; (2) the urea nitrogen concentration has remained relatively constant in both veins; (3) 2 hours after the meal the non-protein nitrogen concentration has increased in both veins, but more conspicuously in the abdominal vein.

Venograms of the abdominal vein.

Blood flow in the vein was stopped by digital pressure. A needle was introduced above and close to the point of occlusion and 20 c.c. of a radio-opaque solution (Pyelosil) injected. The injection was therefore retrograde. X-rays of the right upper quadrant of the abdomen were taken (Fig. 2). The vein starts as a very small tributary passing down over the costal margin (D). It is joined (C) by a larger vein which comes from above and to the right. C represents the point where this deep vein penetrates to the subcutaneous tissue. As the vein proceeds downwards towards the inguinal ligament it becomes very tortuous (A). The surface marking of the umbilicus is also shown (B). It was hoped to take another venogram using a larger volume of pyelosil, but unfortunately the first injection was followed by complete thrombosis of the vein, although pyelosil is usually considered non-irritant to vascular endothelium.

A superficial portion of vein 10 cm. long contained about 12 c.c. of "pyelosil." This portion when emptied of blood filled again in 2 seconds. The blood flow through the abdominal vein was therefore believed to be between 300 and 400 c.c. per minute.

Discussion.

The biochemical results and the venogram make it almost certain that the anterior abdominal wall vein communicated with the portal venous system. In hepatic cirrhosis the intrahepatic obstruction to the portal circulation leads to the opening up of collateral channels. A group forms at the site of the obliterated embryological veins within the falciform ligament (17). These paraumbilical veins connect the left branch of the portal vein with the superficial veins around the umbilicus. In the presence of portal obstruction reasonable mixture of the blood in the right and left branches of the portal vein probably occurs. It is suggested that, in the present patient, some of the blood from the left branch of the portal vein entered a paraumbilical vein and passed in the falciform ligament to emerge through a small paraumbilical hernia and join the superficial epigastric vein. This vein inferiorly joins the great saphenous vein and superiorly communicates with the lateral thoracic veins, tributaries of the axillary vein (3). It is this latter communication which is seen in the venogram (D). Total blood flow through the liver in man is estimated at 1,085 to 1,845 c.c. per minute (2); of this one-quarter to one-eighth is from the hepatic artery. The collateral abdominal vein was estimated to carry 360 c.c. per minute. It can be assumed, therefore, that the anastomotic channel was carrying a half to a quarter of the portal venous blood directly into the right great saphenous vein. This short-circuiting of portal blood and the mixing of the two streams in uncertain proportions prevented studies of hepatic function being included. Observations were confined to study of the contents of the portal collateral vein during absorption from the intestine. Although in 1877 the portal vein was first punctured in animals for this purpose (19) biochemical analysis of portal vein blood does not hitherto seem to have been attempted in man.

In the rat and cat, glucose absorption proceeds more rapidly than that of lævulose (6, 13); similar conclusions have been indirectly reached in man (16). The present observations support this suggestion, the highest concentration of glucose in the abdominal vein preceding that for lævulose, indicating a different rate for the absorption of glucose and lævulose. The interval of 30 minutes between samples prevents more accurate localisation of the actual time of maximum sugar concentration. The mixing of the abdominal collateral with the systemic venous system makes it impossible to calculate the quantities of each sugar absorbed in unit time. The present technique therefore suffers from the same disadvantages as the systemic blood sugar tolerance test, as concentrations rather than absolute quantities are under consideration. It is believed that glucose is absorbed by a phosphorylation mechanism (23) but that the hexose phosphoric acid in the intestinal mucosa is immediately changed into hexose again and that the sugar passes as such into the blood stream. The failure of the ester phosphate concentration in the portal collateral vein to increase during the absorption

of glucose is in keeping with the hypothesis. The absorption of the sugar, moreover, occurred without any significant change in the serum potassium concentrations of either vein.

The partition theory of fat absorption (9, 10, 12) has not been universally accepted (4, 14, 24, 25). It was hoped that observations on the present case might have shown definitely whether fat, split or unsplit, was absorbed into the portal venous system. However, on two occasions fat tolerance tests using triglyceride fat showed neither systemic nor an anterior abdominal vein lipæmia. The fat was introduced directly into the duodenum, the passage of the meal was not hurried and the patient did not have increased fæcal fat concentrations. No explanation can be offered for the failure to demonstrate a venous lipæmia. It has been shown that if neutral fat is given with lipase a portal rather than a systemic lipæmia results (11). In our patient when neutral fat was given with lipase and was presumably hydrolyzed, only then was there some absorption into the portal collateral vein. However, the increase was not sufficiently conspicuous to be entirely conclusive. It might have been more striking if the observations had been continued for three rather than two hours after the fatty meal.

An increase in the nitrogen content of the portal vein during protein digestion has been shown in animals (7, 15, 22). In the present case, although it would have been more satisfactory to have estimated amino acid nitrogen rather than non-protein nitrogen, the increased non-protein nitrogen in the abdominal collateral after a protein meal seemed to confirm these findings, especially as the urea nitrogen concentration remained relatively constant.

Many other studies will spring to mind which would have been attempted if untimely complete thrombosis of the abdominal vein had not occurred. After this mishap fresh veins developed over the right flank, but these new collaterals were multiple, small and not susceptible to puncture. It would also have been more satisfactory if the complication of portal cirrhosis had not been present and if more frequent venous sampling had been possible. The present observations are reported in the hope that others may perhaps be more fortunate when a similar clinical opportunity presents. A portal communication can readily be distinguished from a systemic anastomosis by simultaneous puncture of the vein and an arm vein one hour after a glucose drink. The samples are analysed for glucose. Three other patients with dilated abdominal veins have been studied by this means. In each instance the sugar content of the arm and abdominal vein was identical. In these cases, therefore, the abdominal vein communicated only with the greater venous circulation.

Summary.

1. A patient with hepatic cirrhosis was studied, in whom an enlarged abdominal wall vein communicated with the portal venous system.

2. After oral galactose a higher concentration of galactose was observed in a sample of blood from the anterior abdominal wall vein than in a simultaneous sample from an antecubital vein. Similar results were obtained for lævulose and glucose. The highest concentration of glucose in the abdominal wall vein appeared before that for lævulose. The absorption of these sugars into the abdominal vein was not associated with significant changes in the concentrations of serum ester phosphate or serum potassium.

3. On two occasions after a neutral fat meal had been introduced into the duodenum no increased fat could be shown in the abdominal or in the antecubital vein. When neutral fat was given with lipase there was a suggestive increase in the fat content of the portal collateral vein, but the results were not conclusive.

4. After a protein meal a conspicuous difference existed between the non-protein nitrogen contents of the abdominal vein and that of the systemic vein. Plasma urea nitrogen remained constant.

5. A simple biochemical method of confirming the clinical diagnosis of a portal anastomotic vein is suggested.

APPENDIX.

The following methods were used :—

Serum urea, serum non-protein nitrogen. King, Haslewood and Delory, Lancet, i, 886.

Serum proteins, serum potassium. King, Haslewood, Delory and Beall, Lancet, 1942, i, 207.

Serum total " acid soluble " phosphate and serum inorganic phosphate. King, Biochem. J., 1932, **26**, 292.

The ester phosphate is obtained by subtraction of the inorganic phosphate from the total acid soluble phosphate.

Serum bilirubin. Haslewood and King, Biochem. J., 1937, **31**, 920.

Serum alkaline phosphatase. King and Armstrong, Canad. Med. Assoc. J., 1934, **31**, 376.

Serum cholesterol. Sackett, J. Biol. Chem., 1925, **64**, 203.

Blood sugar. Haslewood and Strookman, Biochem. J., 1939, **33**, 920.

Blood lævulose. Herbert, Biochem. J., 1938, **32**, 815.

Total serum fatty acid and opalescence of serum. Dixon, Drew and Samuel, 1946, in press.

Lipoid phosphorus. Man and Gildea, J. Biol. Chem., 1936, **117**, 183.

Non-phospholipid fatty acid was calculated by the method of Man and Gildea. J. Biol. Chem., 1932, **98**, 43.

Neutral fat was evaluated as glyceryl trioleate corresponding to the non-phospholipoid fatty acid.

Urine galactose. King and Aitken, Lancet, 1940, ii, 543.

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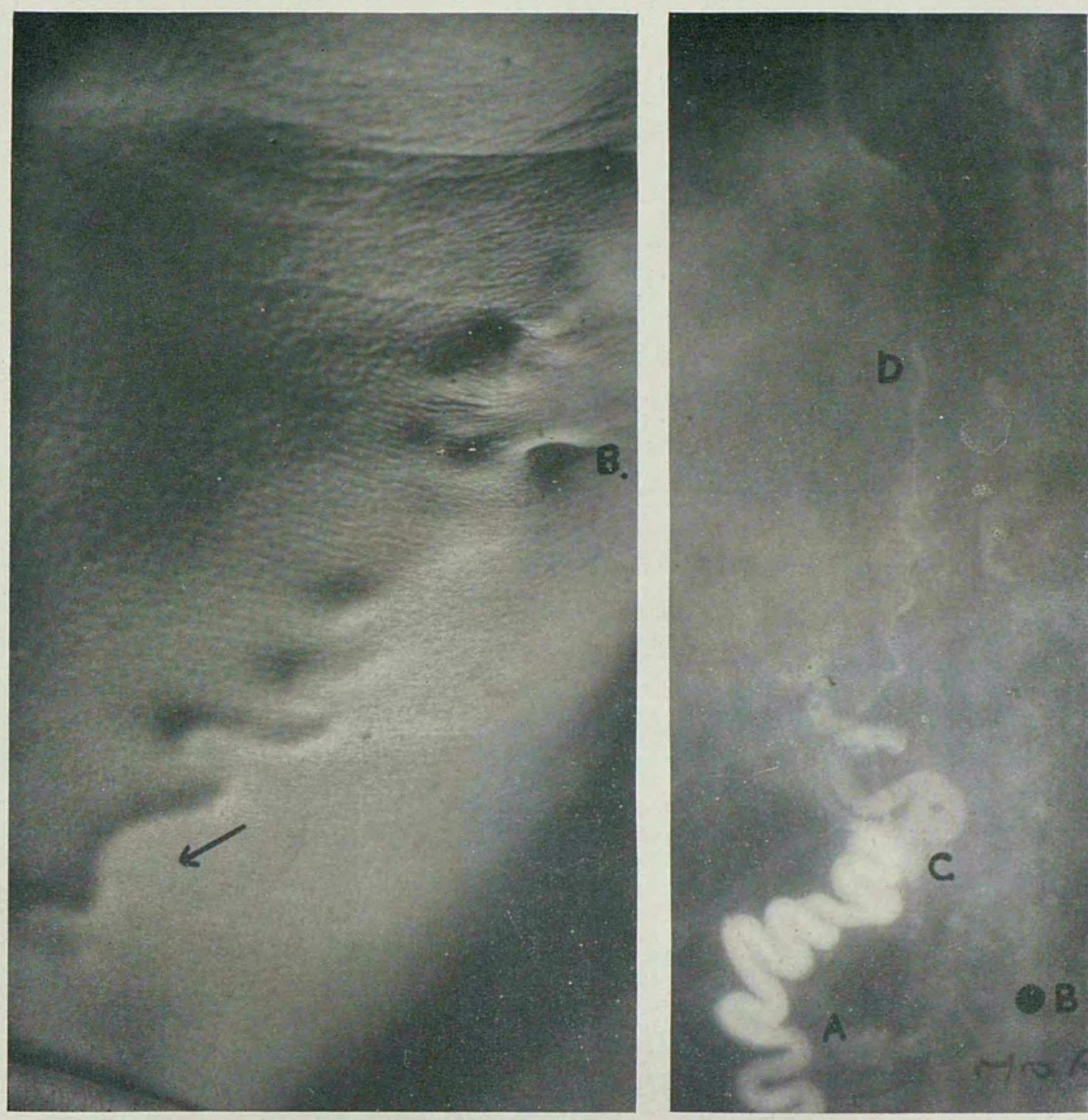

Fig. 1. Fig. 2.

Fig. 1. Photograph of the anterior abdominal wall vein.
Arrow marks direction of blood flow.
B = umbilicus.

Fig. 2. Venogram of anterior abdominal wall vein.
A = tortuous main part of vein (superficial epigastric vein).
B = surface marking of the umbilicus.
C = point of emergence of the vein from the abdomen.
D = the continuation of the vein upwards to join the lateral thoracic vein.

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