sion of cholesterol into pregnanediol (Bloch, 1945), oestrone metabolism (Pearlman *et al.* 1954) and testosterone and cortisone metabolism (Gallagher *et al.* 1954). There are certain biochemical limitations in the valid use of deuterium or tritium, and broad aspects of the problem are discussed by Kamen (1951).

SUMMARY

1. [16-³H]Progesterone was injected intramuscularly into three women in the last trimester of pregnancy and into two oophorectomized-hysterectomized patients who served as a control group. The urinary recovery of tritium in the neutral ketonic and non-ketonic fractions and as 5 β -pregnane-3 α :20 α -diol was determined.

2. Of the radioactivity injected 31 % (average value for all subjects) was recovered in the neutral urinary extracts; the isotope was distributed between the ketonic and non-ketonic fraction in a proportion of about 1:4. 5 β -Pregnane-3 α :20 α -diol was the major radioactive metabolite.

3. The percentage recovery of progesterone as urinary 5 β -pregnane-3 α :20 α -diol was calculated by isotope-dilution procedures to be 6, 15 and 14 % in the pregnant subjects and 27 and 14 % in the oophorectomized-hysterectomized subjects. These findings do not support earlier observations (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953) by conventional analytical methods that the percentage recovery of the metabolite in pregnancy is increased about threefold.

4. The endogenous production of progesterone was similarly calculated to be of the order of 0.25 g./day in late pregnancy.

5. The validity of using [16-³H]progesterone in the study of the intermediary metabolism of the hormone is discussed.

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The Metabolism of Thyroid Hormones

2. DETECTION OF THYROXINE AND TRI-IODOTHYRONINE IN HUMAN PLASMA*

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The introduction of paper chromatography and the availability of radio-iodide enabled Laidlaw (1949) to show that the circulating thyroid hormone was thyroxine and not thyroglobulin or a peptide, a conclusion supported by the work of Taurog,

Chaikoff & Tong (1950) and Rosenberg (1951). After the discovery of tri-iodothyronine (Gross & Pitt-Rivers, 1952*a, b*, 1953*a, b*; Roche, Lissitzky & Michel, 1952*a, b*) it became clear that many of the earlier methods would not have been capable of differentiating between thyroxine and the new substance. The paper-chromatographic techniques

* Part 1: Sprott & MacLagan (1955).

previously used employed for the most part solvents which would not separate the two compounds. The use of new solvent systems, however, has enabled several groups of workers to detect the presence of both compounds in the plasma after the administration of radio-iodide (Dingledine, Pitt-Rivers & Stanbury, 1955; Benua & Dobyns, 1955).

In view of the possibility of radiation artifacts it appeared desirable to conduct parallel studies by non-radioactive methods. The introduction of ceric sulphate-arsenious acid as an extremely sensitive reagent for the detection of thyroxine and related compounds on paper chromatograms has offered an alternative approach (Bowden & MacLagan, 1954; Bowden, MacLagan & Wilkinson, 1955; MacLagan, Bowden & Wilkinson, 1955). The present paper describes the application of this reagent to the study of the iodine-containing compounds of plasma without using radioactive materials. A preliminary account of this work has already appeared (Wilkinson & MacLagan, 1955).

MATERIALS AND METHODS

All glassware was cleaned with chromic acid and immediately before use was rinsed with 0.1N-NaOH and iodine-free water. Water distilled in a glass-lined still proved satisfactory when tested with ceric sulphate and arsenious acid (Bowden *et al.* 1955). 'Deionized' water having a specific resistance greater than 0.8 MΩ/cm., obtained from an Elgastat Deionizer, was used in the preparation of all solutions.

Paper chromatography. Whatman no. 1 paper was used. Chromatograms were developed by the ascending technique with one or more of the following solvent systems: butanol-dioxan-NH₃ (Gross, Leblond, Franklin & Quastel, 1950); isopentanol-NH₃ (Roche, Deltour, Michel & Velez, 1953); butanol-2N-acetic acid; or 2-methylbutan-2-ol-NH₃ (Gleason, 1955). The system containing dioxan was renewed every 2 weeks to avoid complications due to peroxide formation (Taurog, Briggs & Chaikoff, 1952; Owen, McKenzie & Orvis, 1956). After the chromatograms had been dried in an iodine-free atmosphere at room temperature, the positions of the iodine-containing compounds

were located by the ceric sulphate-arsenious acid reagent (Bowden *et al.* 1955).

Thyroxine butyl ester. Thyroxine (50 mg.) was heated under reflux with 80% (v/v) aqueous butanol (20 ml.) and 10N-HCl (0.2 ml.) for 1 hr., after which the solvent was removed under reduced pressure. The residue was suspended in 0.05N-Na₂CO₃ (3 ml.) and continuously extracted with ether. Evaporation of the solvent, followed by recrystallization of the residue from ethyl acetate, gave thyroxine butyl ester, 20 mg. m.p. 89° (decomp.) (Found: C, 27.8; H, 2.7; N, 1.8; I, 60.0. C₁₅H₁₉O₄NI₂ requires C, 27.4; H, 2.3; N, 1.7; I, 60.9%). It gave a negative ninhydrin test in solution, though on paper sprayed with ninhydrin reagent it produced a pink spot. When sprayed with diazotized sulphanilic acid solution a spot on paper gave a red colour, indicating the presence of a free hydroxyl group. The same product was obtained when 10% (v/v) aqueous butanol was used as solvent.

3:5-Di-iodo-L-tyrosine butyl ester. 3:5-Di-iodo-L-tyrosine (2 g.) was heated under reflux with butanol (90 ml.) and 10N-HCl (10 ml.). The residue obtained by evaporation under reduced pressure was suspended in 0.05N-Na₂CO₃ (10 ml.) and continuously extracted with ether. The ether-insoluble material consisted of unchanged di-iodotyrosine. The ether was evaporated and the residue crystallized from ethanol (charcoal). The ester (1.0 g.) had m.p. 135° (Found: C, 32.3; H, 3.8; N, 2.7; I, 52.5. C₁₈H₁₇O₃NI₂ requires C, 31.9; H, 3.5; N, 2.9; I, 51.8%). It gave a positive diazotized sulphanilic acid test and a negative ninhydrin test.

By similar means thyroxine was converted into its methyl and ethyl esters and tri-iodothyronine into its butyl ester. These substances were not isolated but their presence was demonstrated chromatographically. Hydrolysis with NaOH regenerated the parent amino acids. The *R_F* values of the esters are listed in Table 1.

Typical procedure for the extraction of thyroxine and tri-iodothyronine from plasma

Blood was collected by venepuncture and the plasma separated within 1-2 hr.; oxalate or heparin was used as anticoagulant. The plasma (10 ml.) was shaken with methanol (24 ml.) for 1-2 min. (Brown & Jackson, 1954). The protein was removed by centrifuging and the supernatant was separated. The protein precipitate was washed twice with 70% (v/v) aqueous methanol (10 ml.). The combined washings and supernatant were evaporated to

Table 1. *R_F* values of esters of di-iodotyrosine and iodothyronines

Ascending method on Whatman no. 1 paper. For references to composition of solvent mixtures see Methods.

Amino acids	Esters	<i>R_F</i> values in		
		Butanol-dioxan-2N-NH ₃	2-Methylbutan-2-ol-2N-NH ₃	isoPentanol-2N-NH ₃
3:5-Di-iodotyrosine	—	0.18	0.02	0.02
3:5-Di-iodotyrosine	Butyl	0.86	0.75	0.83
3:5-Di-iodothyronine	—	0.72	0.58	0.50
3:5-Di-iodothyronine	Butyl	0.97	0.93	0.95
3:5:3'-Tri-iodothyronine	—	0.70	0.54	0.39
3:5:3'-Tri-iodothyronine	Butyl	0.95	0.89	0.89
Thyroxine	—	0.59	0.32	0.24
Thyroxine	Methyl	0.86	0.84	0.87
Thyroxine	Ethyl	0.91	0.87	0.94
Thyroxine	Butyl	0.90	0.89	0.85

dryness at $20 \pm 5^\circ$ at 2–3 mm. The residue was incubated with 0.06M-phosphate buffer of pH 7.4 (2 ml.) and a solution of crystalline trypsin (0.2 mg.) in 0.05N-HCl (0.2 ml.) at 37° for at least 2 hr., or overnight if convenient. Methanol (4 ml.) and light petroleum (b.p. $40\text{--}60^\circ$) (6 ml.) were added; the mixture was shaken vigorously and centrifuged and the upper layer was discarded. The flask was rinsed with light petroleum (6 ml.), which was then used to re-extract the lower layer in the centrifuge tube. This process was repeated twice until the aqueous methanolic layer had been extracted at least four times. The lower layer was then returned to the flask, the small amount of solid being rinsed with 70% (v/v) methanol and discarded. The combined methanolic layer was evaporated at $20 \pm 5^\circ$ at 2–3 mm. The residue was dissolved in 0.02N-HCl (3 ml.) (the pH of the resulting solution was about 5) and extracted three times with butanol (3 ml., 2 ml., 2 ml.) below 15° : separation of the layers was completed by centrifuging. The combined extract was washed with water (6 ml.) and evaporated at $15 \pm 5^\circ$ at 2–3 mm. as rapidly as possible. The gummy residue (about 0.2 mg.) was dissolved in 0.02N- Na_2CO_3 (2 ml.) and the solution twice extracted with ether (6 ml.). The aqueous layer was adjusted to pH 4–5 with 0.02N-HCl (2 ml.) and extracted as above with butanol. The extract was washed with water and evaporated as described above. The residue was dissolved in 50 μl . of butanol and 10 μl . portions (equivalent to 2 ml. of plasma) were chromatographed, except in certain cases of hypothyroidism when larger amounts of the extracts (up to 33 μl .) were used. Thyroxine and tri-iodothyronine markers (0.05–0.2 μg .) were always run alongside the extracts. Stained spots were assessed visually within 1 hr. (Table 2).

Determination of thyroxine and tri-iodothyronine on paper chromatograms by elution method

Rectangles of paper containing the spots of thyroxine and tri-iodothyronine were cut from the dried unstained paper after location by a stained marker strip. Each was folded and immersed in water (3 ml.) and 0.2N-arsenious acid (Leffler, 1954) (2 ml.) at 25° for 20 min. At 30 sec.

intervals 0.02N-ceric sulphate (Leffler, 1954) (1 ml.) was added to each tube, which was then shaken for 1 hr. at 25° . Each solution was then decanted from the paper and the percentage transmission measured at 30 sec. intervals in an EEL absorptiometer (Ilford 601 violet filter) adjusted for 100% transmission with water. Calibration curves are shown in Fig. 1.

RESULTS

Stability of solutions of thyroxine and tri-iodothyronine

In aqueous 0.02N-sodium carbonate at room temperature thyroxine (1 mg./10 ml.) yielded in 2–3 days iodide and a substance which on paper chromatography behaves like tri-iodothyronine (Wilkinson, Sprott, Bowden & MacLagan, 1954). In 50% (v/v) aqueous propylene glycol adjusted to pH 8–9 with diethanolamine, thyroxine underwent similar decomposition more slowly; so that iodide was detected after about 10 days. A 0.0025N-solution of NaOH in 99% aqueous butanol also caused deiodination. At higher temperatures, the same type of decomposition occurred more rapidly. Tri-iodothyronine behaved similarly in alkaline solution, and after several months iodide only was detectable. In acid alcohols, however, esterification took place. Fast-moving spots (R_f 0.90–0.95) were observed when solutions of either substance in 0.1N-HCl in 99% butanol were stored at $20^\circ + 5^\circ$ for prolonged periods and chromatographed in butanol–dioxan–ammonia. This process eventually went to completion. When the solutions were heated at 100° , free thyroxine or tri-iodothyronine could not be detected after 1 hr. The products were proved to be the butyl esters by comparison of R_f values with authentic specimens. On treatment with 0.05N-NaOH at 100° for 10 min. the esters yielded thyroxine and tri-iodothyronine respectively. Di-iodotyrosine was also converted into its butyl ester in acid butanol, and other alcohols gave corresponding esters with each of the iodinated amino acids studied. It is clear, therefore, that on storage in aqueous butanol containing hydrochloric acid esterification is liable to occur. The proportions of water and butanol did not seem to have any effect on the reaction, for esterification occurred almost as readily in a solution of butanol in water as in water-saturated butanol.

To exclude esterification as a source of artifacts the pH was kept above 4 during all stages of the extraction. Under these conditions no evidence of esterification was detected.

Recovery of added thyroxine

The extract prepared from 5 ml. of plasma to which 0.1 μg . of thyroxine and 0.1 μg . of tri-iodothyronine had been added gave satisfactory chromatograms. The sizes and positions of the spots

Fig. 1. Typical calibration curves for the determination of thyroxine (O) and tri-iodothyronine (●) by elution from paper chromatograms with the ceric sulphate-arsenious acid reagent.

produced after treatment with the ceric sulphate-arsenious acid reagent were comparable with those obtained from marker solutions containing similar quantities of thyroxine and tri-iodothyronine.

The quantitative recovery of added thyroxine was measured in two stages: (1) from plasma into the final butanol extract; (2) from the butanol extract to the developed chromatogram and colorimetric determination. The recovery during the first stage was determined by adding 0.2–0.4 $\mu\text{g.}$ of ^{131}I -labelled thyroxine to plasma and comparing the counting rates of the plasma and the final extracts. After allowance was made for radioactive decay, the recoveries in the five experiments were 90, 90, 97, 91 and 87 %.

During the second stage thyroxine and tri-iodothyronine (0.1 or 0.2 $\mu\text{g.}$) were added to extracts equivalent to 2 ml. of plasma which were chromatographed, and the iodine compounds were determined by the elution method. Duplicate results indicated good reproducibility and the recoveries of added material ranged from 70 to 110 % (mean 87 ± 10 %, 16 expts.) for thyroxine and from 50 to 90 % (mean 71 ± 11 %, 16 expts.) for tri-iodothyronine.

Clinical results by visual assessment

The results obtained during the examination of the plasma of a number of individuals in various states of thyroidal activity are summarized in Table 2. The euthyroid group includes some

Table 2. *Plasma thyronines determined by visual assessment method*

0 = Not detected; + = detectable with difficulty or present in small amount; ++ = easily detectable; +++ = easily detectable in relatively large amounts.

Subject			State	Thyroxine	Tri-iodothyronine
No.	Sex	Age			
1	F.	18	Thyrototoxic	+++	++
2	F.	37	Thyrototoxic	++	++
3	F.	39	Thyrototoxic	+	+++
4	F.	70	Thyrototoxic	++	+
5	F.	50	Thyrototoxic	+	+
6	F.	52	Thyrototoxic	+	+++
7	M.	23	Thyrototoxic	+	++
8	F.	37	Thyrototoxic	++	+++
9	F.	35	Thyrototoxic	+	++
10	F.	42	Thyrototoxic	++	+
11	F.	63	Thyrototoxic	+++	+
12	F.	50	Thyrototoxic	++	+
13	M.	42	Thyrototoxic	+++	+
14	F.	58	Thyrototoxic	++	+++
15	M.	52	Thyrototoxic	++	+++
16	F.	32	Thyrototoxic	++	+
17	F.	36	Hyperthyroid	+++	+
18	F.	24	Euthyroid	+	+
19	F.	20	Euthyroid	+	+
20	F.	30	Euthyroid	+	+
21	F.	61	Euthyroid	+	++
22	M.	44	Euthyroid	0	++
23	M.	33	Euthyroid	+	++
24	F.	32	Euthyroid	++	0
25	F.	8	Euthyroid	++	+
26	F.	21	Euthyroid	++	+
27	F.	56	Hypophysectomy regeneration	++	+
28	F.	64	Euthyroid (non-toxic goitre)	+	0
29	M.	14	Euthyroid (non-toxic goitre)	+	+
30	F.	34	Hypothyroid	++	0
31	F.	2	Hypothyroid	0	0
32	F.	53	Hypothyroid	+	0
33	M.	42	Hypothyroid	+	0
34	F.	62	Myxoedema	0	0
35	—	—	Myxoedema	0	0
36	F.	65	Myxoedema	0	0
37	F.	54	Myxoedema	0	0
38	M.	42	Myxoedema	0	0
39	—	—	Myxoedema	0	0
40	F.	67	Myxoedema	+	0
41	F.	67	Myxoedema	0	0
42	F.	57	Hypothyroid	++	0
43	F.	28	Hypothyroid	++	0

members of the laboratory staff who were in good health, as well as hospital patients under investigation for conditions not involving the thyroid gland. Tri-iodothyronine was detected in the plasma of all members of the thyrotoxic group and nearly all the normal controls, but was not found in any of the 14 hypothyroid subjects.

The possibility that thyroxine was partly converted into the tri-iodo compound during the extraction procedure was eliminated as follows: (a) radiothyroxine and carrier tri-iodothyronine were added to normal plasma, which was then extracted and the extracts were chromatographed. Radioautographs of the chromatogram showed single thyroxine spots: no labelled tri-iodothyronine was detected. (b) A large excess (0.4 μ g.) of non-radioactive thyroxine was added to the plasma of a hypothyroid patient previously shown not to contain tri-iodothyronine. The chromatogram showed only thyroxine.

In addition to the demonstration that the spots on the chromatograms were coincident with similar spots containing added thyroxine and tri-iodothyronine, identification was confirmed by developing the chromatograms in a number of different solvent systems. No known compound has the same R_F as thyroxine in each of the systems used, but 3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)-phenylacetic acid has very similar chromatographic properties to tri-iodothyronine. As the presence of this substance has been demonstrated in biological systems (Roche, Michel & Jouan, 1957; Albright, Larson, Tomita & Lardy, 1956) it was desirable to distinguish between the two compounds. At the suggestion of Professor S. Lissitzky the behaviour of the two compounds on paper ionophoresis was examined. In barbitone buffer of pH 8.6 at a potential of 150 v tri-iodothyronine did not move in 16 hr., whereas 3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)-phenylacetic acid moved 4.6 cm. towards the anode in 4 hr. Under similar conditions

thyroxine moved about 0.2 cm. in 16 hr. and 3:5-di-iodo-4-(4-hydroxy-3-iodophenoxy)-phenylacetic acid 2.8 cm. in 4 hr. in the same direction. Extracts of plasma of two cases of thyrotoxicosis were chromatographed in 2-methylbutan-2-ol-ammonia for 16 hr.; the papers were dried and placed in an electrophoresis tank such that the direction of the electric potential was perpendicular to the chromatographic flow. After 4 hr. at 150 v the spots of R_F 0.54 had not moved in response to the electric potential and consequently were those of tri-iodothyronine.

Several plasma extracts contained an unidentified brown pigment (R_F in 2-methylbutan-2-ol- NH_3 , 0.68) which, although easily distinguished from tri-iodothyronine (R_F 0.54) in this solvent, overlapped this region in others. This substance fluoresced yellow-brown in ultraviolet light and interfered with the preparation of permanent records by ultraviolet photography (Bowden *et al.* 1955). In marked contrast with the behaviour of the iodothyronines and iodyrosines it decolorized the ceric sulphate-arsenious acid reagent only after 12–24 hr.

In one of the hypothyroid patients and in two of the normal individuals small amounts of an unknown iodine-containing component were detected. It had an R_F value of 0.54–0.58 in *isopentanol*- NH_3 and 0.60 in 2-methylbutan-2-ol- NH_3 . Unfortunately there was not sufficient material for further investigation.

Clinical results by quantitative elution method

The results obtained from 10 patients by this procedure are shown in Table 3. Although this small series is an inadequate test of the method it will be seen that euthyroid patients have plasma thyroxine values up to 5 μ g./100 ml. and plasma tri-iodothyronine values up to 0.5 μ g./100 ml. The high values obtained in the first 5 cases were expected. In addition the distinction between

Table 3. *Plasma thyronines determined by elution method*

Subject			State	Thyroxine (μ g./100 ml.)	Tri-iodothyronine (μ g./100 ml.)
No.	Sex	Age			
12	F.	50	Thyrotoxic	5	1
13	M.	42	Thyrotoxic	10	<1
15	F.	52	Thyrotoxic	5	4
43	F.	28	Hypothyroid (after treatment with tri-iodothyronine)	2.5	9
44	M.	45	Patient had taken thyroid extract	32	4.5
44	M.	45	After 6 weeks in hospital without thyroid medication	2.5	<0.5
45	F.	33	Hypothyroid	1.5	<0.5
46	M.	21	Carcinoma of thyroid	4.5	<0.5
47	M.	32	Euthyroid	4.5	0.5
48	F.	31	Euthyroid	5	0.5
49	F.	44	Hypothyroid	2.5	<0.5

case 43 (tri-iodothyronine therapy) and case 44 (treated with thyroid extract) is clearly shown. The procedure was too time-consuming for routine use, but it may have a place in special investigations.

DISCUSSION

The results summarized in Table 2 show that tri-iodothyronine is a true component of the organic iodine fraction of normal and thyrotoxic plasma and that it is somewhat more easily detectable in the latter than in the former. This is in complete agreement with earlier work with radio-iodine (Dingledine *et al.* 1955; Benua & Dobyns, 1955). The latter authors suggested that the amount of circulating tri-iodothyronine was probably related to the degree of thyrotoxicosis. The possibility that the tri-iodothyronine detected by these workers was a radiation artifact could not entirely be discounted, though Benua, Dobyns & Ninmer (1955), who found it as early as 1 hr. after the administration of radio-iodide, suggest that radiation damage is unlikely to be responsible. The present work confirms the conclusion drawn from the radioactive investigations, since there can be no question of interference with the gland.

Tri-iodothyronine was uniformly absent in the fourteen hypothyroid patients, though thyroxine was found in six of them. Eight of the patients in this group suffered from myxoedema and in one of these thyroxine was detected in the plasma. In this subject it is possible that the myxoedema may have arisen as a result of a peripheral failure to convert thyroxine into tri-iodothyronine as reported by Kurland, Hamolsky & Freedberg (1955).

The quantitative results shown in Table 3 suggest that the method could be used to demonstrate variations in the proportions of thyroxine and tri-iodothyronine in the plasma. Such variations may account for discrepancies which have occasionally been observed between the plasma protein-bound iodine levels and the thyroid status of the individual (Mowbray & Tickner, 1952; Schultz, Sandhaus, Demorest & Zieve, 1954; Vannotti, 1957).

SUMMARY

1. A method for extracting the iodine-containing components of plasma has been described. Paper chromatography combined with paper ionophoresis has enabled the circulating substances to be identified as thyroxine and tri-iodothyronine.

2. Thyroxine and tri-iodothyronine have been detected in the plasma of sixteen thyrotoxic patients and fifteen normal or euthyroid subjects, but tri-iodothyronine was not found in the plasma of any of the seventeen hypothyroid patients.

3. The stability of solutions of thyroxine and tri-iodothyronine in a number of solvent systems has been studied. In aqueous alkaline solution both substances slowly liberated iodide, but in acid alcoholic solutions both compounds were rapidly esterified even in the presence of relatively large concentrations of water.

4. A method devised for the determination of thyroxine and tri-iodothyronine on paper chromatograms has been used to estimate the circulating levels of these substances in ten patients.

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The Determination of Ketone Bodies

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In the determination of ketone bodies it is customary to convert acetoacetic acid and β -hydroxybutyric acid into acetone for estimation. This may be brought about during distillation (Shaffer, 1908-09; Shipley & Long, 1938; Weichselbaum & Somogyi, 1941) or by refluxing or heating in pressure bottles (Van Slyke, 1917; Greenberg & Lester, 1944; Michaels, Margen, Liebert & Kinsell, 1951). When distillation is employed preformed acetone plus acetoacetic acid may be estimated separately from β -hydroxybutyric acid in the one blood or urine filtrate; this is not possible when refluxing or pressure bottles are used. However, the latter methods permit greater reproducibility in the estimation of β -hydroxybutyric acid, since the acid-dichromate reagent used for the oxidation is maintained at constant concentration.

The present method combines the advantages of both procedures by using a combined reflux and distillation apparatus. Acetone in the distillates is converted into the 2:4-dinitrophenylhydrazone, which is extracted with carbon tetrachloride and estimated colorimetrically by a modification of the method of Greenberg & Lester (1944).

MATERIALS AND METHODS

Reagents. Sulphuric acid, 20N; potassium dichromate, 2.5% (w/v); freshly filtered 2:4-dinitrophenylhydrazine, 0.1% (w/v) in 4N-HCl; carbon tetrachloride; NaOH, 0.5N; barium hydroxide, approx. 0.3N; zinc sulphate, approx. 5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (10 ml. of the barium hydroxide solution added drop by drop with phenolphthalein as indicator, should neutralize exactly 10 ml. of the ZnSO_4 solution). Distilled water is used throughout the determination. All reagents are of A.R. grade.

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Preparation of standard solutions

Acetone. A.R. acetone was redistilled three times, only the middle fraction boiling at 56.5° being retained. Standard solutions were prepared by weighing up to 1 g. of acetone in a stoppered tube and dissolving it in water. The possibility of loss of acetone due to volatilization was overcome by suspending the stoppered tube beneath water in a 2 l. volumetric flask and removing the stopper by means of an attached thread. This solution was made up to 2 l. and diluted as required.

Acetoacetic acid. Widmark (1920-21) found that acetoacetic acid in acid solution underwent complete decomposition to acetone, the determination of which could be used as a means of estimating quantitatively the acetoacetic acid. Accordingly, a solution of sodium acetoacetate, prepared by the method of Ljunggren (1924), was standardized with the present method.

β -Hydroxybutyric acid. The calcium-zinc salt of β -hydroxybutyric acid was prepared according to Shaffer & Marriott (1913-14) and Blunden (1938). Colourless needles and plates were obtained (m.p. $247.5\text{--}248.5^\circ$ uncorr. with

Fig. 1. Combined reflux and distillation apparatus.