CCXI. A METHOD OF DETERMINATION OF SOME BIOLOGICALLY IMPORTANT ALDEHYDES AND KETONES, WITH SPECIAL REFERENCE TO PYRUVIC ACID AND METHYLGLYOXAL.

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I. INTRODUCTION.

THE importance of various types of unsaturation in molecules in intermediary metabolism is a subject to which attention is being increasingly devoted. During the study of some of the intermediary mechanisms of carbohydrate metabolism in the liver we have been interested in one type of unsaturated linkage, namely that of the carbonyl group. Aldehydes and ketones possess, as is well known, the capacity of forming hydrazones and osazones, which capacity is sometimes used in their identification and estimation. Another property is their power of forming compounds with bisulphites. This property has long been used as a method of estimating acetaldehyde [Ripper, 1900]. That this method can be used for other aldehydes and ketones was shown by Cook [1930] who based on it a method for the estimation of pyruvic acid.

We have extended the method to other aldehydes and ketones with, we feel, some measure of success and can utilise the method, for example, for the determination of a mixture of pyruvic acid with acetaldehyde or methylglyoxal in a single sample. The method shares the disadvantage of unspecificity with the other methods of estimation of carbonyl compounds, there being inherently no specific method of estimation for a mixture of two carbonyl compounds except in so far as the hydrazones or osazones of acids may be separated from those of non-acidic compounds by their solubility in sodium carbonate [Dakin and Dudley, 1913].

Owing to the considerable attention focused on the position of methylglyoxal in intermediary metabolism numerous methods have been proposed for its estimation. As will be shown later the question of the estimation of methylglyoxal in tissues is rather a difficult one.

Wendel [1932] has given a very complete account of the methods that have been used for the estimation of pyruvic acid. In addition to pure pyruvic acid we have devoted some attention to the subject of the polymerides of this acid.

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We feel that our method offers a great advantage in the rapidity with which estimations can be carried out with the minimum of apparatus and the necessity of only one standard solution.

II. PRINCIPLE AND EXPERIMENTAL DETAILS OF ESTIMATIONS OF A SINGLE SUBSTANCE.

In the presence of excess of sodium bisulphite the aldehydes and ketones studied react at room temperature to form bisulphite compounds. The principle of the method is to remove the excess bisulphite from the solution by adding the required amount of iodine. The bisulphite compounds are then hydrolysed by saturating the solution with sodium bicarbonate, and the liberated bisulphite is titrated with a dilute standard iodine solution. Thus in general 1 cc. of *N* iodine is equivalent to half the molecular weight of the aldehyde or ketone expressed in mg. The principle is similar to that described by Clausen [1922] and amplified by Friedemann, Cotonio and Shaffer [1927].

The conditions which we have found most suitable are as follows. A 5 cc. aliquot of the solution to be estimated is added to 1.0 cc. of approximately *M* sodium bisulphite solution. The solution is allowed to stand for at least 15 minutes and then a few drops of starch solution are added. The excess bisulphite is then removed by running in approximately *N*/10 iodine solution (conveniently from a burette) until the blue colour indicates the presence of a slight excess of iodine. The blue colour is discharged by the addition of a few drops of approximately *N*/100 sodium thiosulphate solution. The end-point is then adjusted to a faint blue colour by means of the dilute standard iodine solution (*N*/100, *N*/200 or *N*/500 according to the concentration of the carbonyl compound). 5 cc. of an aqueous suspension of sodium bicarbonate are then added and the standard iodine again run in until the blue colour of the indicator is of the same intensity as it was before the addition of the bicarbonate.

It is important that the standard iodine should be freshly diluted with oxygen-free water and also that the bicarbonate suspension should be free from oxygen. The latter end is readily achieved by blowing a stream of carbon dioxide through it for a few minutes immediately before use.

In most cases the reaction is stoichiometric, but where this is not the case an empirical relationship has been found as described below.

With most of the substances studied the reaction between the carbonyl compound and the sodium bisulphite is complete within a very few minutes. With certain substances (notably acetaldehyde and methylglyoxal) it is necessary to allow the solution to stand for 15 minutes.

It is also important when dealing with solutions of biological origin that the reaction should be slightly acid before adding the bisulphite. (See note in section VIII on the effect of glucose on the titration.) The acidity should not, however, be greater than *N*/10, as low results are obtained in strongly acid solution.

With methylglyoxal the acidity of the 5 cc. aliquot should not be greater than *N*/50. The acid most likely to be present in solutions of biological origin is trichloroacetic acid. Table I shows the effect of various concentrations of this acid on the estimation of methylglyoxal.

Table I.

Mg. methylglyoxal		Normality of trichloroacetic acid	% of theory
Added	Found		
1.16	1.07	0.122	92
1.16	1.12	0.061	97
1.16	1.16	0.024	100
0.436	0.419	0.122	96
0.436	0.436	0.024	100

III. PRINCIPLE AND EXPERIMENTAL DETAILS OF THE ESTIMATION OF A MIXTURE OF AN ALKALI-STABLE AND AN ALKALI-LABILE SUBSTANCE.

While certain of the biologically important carbonyl compounds are both non-volatile and stable towards hot dilute alkali, the majority disappear nearly completely when the solution is heated to 100° with dilute alkali in an unstoppered flask. We have found it possible to make use of this difference in properties to effect an approximate estimation of the components of a mixture of such an alkali-stable compound with one which is destroyed by alkali.

With the alkali-stable compound which we have investigated, namely pyruvic acid, the percentage remaining after treatment with alkali under the conditions described below is 93 %, while with the alkali-labile compounds (methylglyoxal, acetaldehyde, glyceraldehyde, dihydroxyacetone *etc.*) a few per cent. only remain. The method is to titrate an aliquot in the usual manner and to titrate another aliquot after heating with alkali.

It is found that the fraction remaining undestroyed is independent of the initial concentration in the case of the stable compound, and approximately a rectangular hyperbolic function of it in the case of the labile compounds. Thus if y mg. of the labile compound are present, and f is the fraction remaining after treatment with alkali,

$$yf = K.$$

The constant K has been determined for a number of compounds (see section V).

Let F = fraction of the stable substance remaining after treatment with alkali,

x = mg. of the stable substance originally present,

1 cc. $N/10$ I = p mg. alkali-labile substance,

1 cc. = q mg. alkali-stable substance.

Then if the titrations before and after the alkali treatment are T_1 and T_2 cc. respectively,

$$x = \frac{q}{F} \left(T_2 - \frac{K}{p} \right)$$

$$y = p \left(T_1 - \frac{T_2}{F} \right) + \frac{K}{F}.$$

The conditions we have chosen for the treatment with alkali are as follows. A 5 cc. aliquot of the solution is placed in a 125 cc. Erlenmeyer flask together with a drop of thymolphthalein solution. 3 cc. of N NaOH are then added in excess of the amount required just to cause the solution to turn blue. The flask is then placed for 1 hour on the boiling water-bath. At the end of this time the contents of the flask are rendered slightly acid with N H_2SO_4 . The exact concentration of alkali and the exact time of heating are not of great importance, at least in the case of methylglyoxal. Table II shows the effect of varying these factors.

Table II.

5 cc. aliquots of methylglyoxal, each containing 0.30 mg.

cc. N NaOH	Time of heating (hours)	Fraction remaining
0.5	1.0	0.045
1.0	1.0	0.030
2.0	1.0	0.027
4.0	1.0	0.030
3.0	0.5	0.033
3.0	1.0	0.034
3.0	1.5	0.034
3.0	2.0	0.033

IV. EXAMPLES OF THE ESTIMATION OF A SINGLE SUBSTANCE.

(1) *Pyruvic acid*.

Pyruvic acid (B.D.H.) was repeatedly distilled *in vacuo* to remove the α -keto- γ -valerolactone- γ -carboxylic acid which may constitute up to 30 % of the average commercial sample of pyruvic acid. The acid was frozen out and the crystals, M.P. 10° (approx.), dissolved in water. No attempt was made to obtain this very deliquescent substance in an anhydrous condition, and the stock solutions were standardised by the method of Wieland [1923]. The results obtained by this method agreed perfectly with those obtained by titration with alkali.

Table III shows the results of bisulphite titrations of the stock solutions.

Table III.

1 cc. N/10 I $\equiv$ 4.4 mg. pyruvic acid.

Mg. pyruvic acid		% yield
Used	Found	
22.0	22.0	100
5.58	5.49	98
2.79	2.88	103
2.20	2.20	100
1.39	1.35	97
0.697	0.700	100
0.349	0.351	101
0.221	0.225	102

Note on certain degradation products of pyruvic acid.

(a) α -Keto- γ -valerolactone- γ -carboxylic acid. As first observed by Wolff [1899] pyruvic acid undergoes spontaneous condensation to this substance. As indicated above commercial samples of pyruvic acid may contain up to 30 % of the lactone. Other decomposition products are also present, as indicated by the odour and colour of old samples of the acid. In order to determine the behaviour of these impurities in the method of estimation we have studied the residue which remains after distillation of pyruvic acid *in vacuo*. This syrupy residue crystallises after standing for a few hours *in vacuo* over quicklime [Wolff, 1901], and consists largely of α -keto- γ -valerolactone- γ -carboxylic acid. A solution of the dry substance takes up iodine in the presence of sodium bicarbonate. The end-point is ill-defined, but the amount of standard iodine taken up corresponds to about half that consumed by an equal weight of pyruvic acid. The titre is not increased by previously allowing the solution to stand with sodium bisulphite.

We have observed that dilute solutions of commercial pyruvic acid give a higher bisulphite titration after long standing than when freshly made up. This is probably due to hydrolysis of the lactone to pyruvic acid. Thus a solution with an initial titre corresponding to 28.0 mg./cc. of pyruvic acid gave after standing for 4 months at about 4° in the presence of toluene a titre corresponding to 33.1 mg./cc. Thus it appears that a convenient way of storing pure pyruvic acid is as the aqueous solution of the free acid.

(b) The so-called "parapyruvate." The so-called parapyruvates are formed when neutral or slightly alkaline aqueous solutions of pyruvates are boiled or allowed to stand for long periods at room temperature. We would like to point out that statements have been made that a substance which, on alkaline hydrolysis, reverts to pyruvic acid is formed when a neutral pyruvate solution is incubated for long periods with bacteria [see Quastel and Wooldridge, 1929, p. 133]. The observations of Wolff [1899] and de Jong [1901; 1902] indicate that the so-called *meta*- and *parapyruvates* are formed under the same conditions without the bacteria.

Barium *parapyruvate* was prepared by allowing a saturated solution of crystalline barium pyruvate to stand for several days at room temperature [de Jong, 1902]. The amorphous white solid which separated was washed thoroughly by repeated shaking with water and air-dried to constant weight. The composition of the product thus obtained is, according to de Jong, $C_6H_6O_6Ba \cdot 4H_2O$. The barium was removed by shaking an accurately weighed amount with a slight excess of dilute sulphuric acid, filtering off the barium sulphate, and making up the filtrate and washings to a known volume with water.

The *parapyruvic acid* thus prepared was found to have a capacity of uniting with bisulphite of approximately one-third that of the equivalent amount of pyruvic acid. On this basis, that 1 cc. $N/10$ I $\equiv$ 13.2 mg. of *parapyruvic acid*¹, the figures given in Table IV were obtained.

Table IV.

mg. <i>parapyruvic acid</i> *	
Used	Found
15.3	16.5
7.6	8.2
3.8	3.8
1.9	1.8
0.95	0.80

* Expressed as pyruvic acid.

When aliquots of the solution were heated with alkali as described in section III a strong yellow colour developed. It was not possible to reach a sharp end-point in a bisulphite titration after this treatment. However, the approximate titrations obtained were uniformly higher than before the alkali-treatment.

(2) *Methylglyoxal*.

Methylglyoxal was prepared by the method of Fischer and Taube [1924], immediately redistilled *in vacuo* and weighed by transferring it to a tared glass-stoppered flask of water as soon as it melted. The solutions thus obtained were perfectly clear. The empirical relationship between the amount of iodine used in a bisulphite titration and the weight of methylglyoxal present was perfectly consistent with different preparations. We have, however, been unable to reproduce quantitatively the results obtained by Kuhn and Heckscher [1926] who describe an empirical method of estimating methylglyoxal by oxidation with iodine in a sodium bicarbonate-carbonate buffer mixture. There seemed to us to be an ambiguity in the phrase "eines Gemisches von 0.2 molarer Na_2CO_3 - und 0.2 molarer $NaHCO_3$ -Lösung." We therefore carried out estimations on our stock solutions according to their method, using (a) a mixture containing 0.2 mols. Na_2CO_3 and 0.2 mols. $NaHCO_3$ in one litre, and (b) the above mixture diluted to twice its volume with water.

Estimations carried out on a solution containing 9.875 mg. of methylglyoxal yielded the following results:

Mixture (a) 13.20, 13.23, 13.23 mg.
 „ (b) 12.11, 11.75, 11.78 mg.

When methylglyoxal is titrated according to our method, the iodine titre does not correspond to a stoichiometric union of either one or two molecules of bisulphite with one molecule of methylglyoxal, but to an intermediate

¹ Expressed as pyruvic acid.

value. For quantities between 0.2 and 2.0 mg. of methylglyoxal (in 5 cc.) a linear relationship holds between the titre and the amount of methylglyoxal, 1 cc. $N/10$ I being equivalent to 2.85 mg. of methylglyoxal. Over this range the end-points are perfectly sharp, but with greater concentrations of methylglyoxal this is not the case, and the magnitude of the titre indicates the union of more bisulphite with the methylglyoxal.

The results of some estimations are shown in Table V.

Table V.

1 cc. $N/10$ I $\equiv$ 2.85 mg. methylglyoxal.

Mg. methylglyoxal		% yield
Used	Found	
1.99	1.91	96
1.44	1.45	101
0.996	1.01	101
0.718	0.738	103
0.498	0.491	99
0.249	0.251	101

This relationship being an empirical one, it is important that the conditions which we have described should be strictly observed. The volume of the solution of methylglyoxal is 5.0 cc., and the sodium bisulphite is added as 1.0 cc. of M solution. The above relationship does not hold when the concentration of bisulphite differs greatly from that specified.

It is also important that the normality of the methylglyoxal solution with respect to acid should not be greater than $N/50$ (Table I).

(3) Dihydroxyacetone.

Dihydroxyacetone was prepared by oxidising glycerol through the agency of *Acetobacter suboxydans*¹. The dihydroxyacetone was isolated from the culture medium according to the directions of Bertrand [1898]. The dimeric compound was purified by washing with alcohol and then distilled at a pressure of 0.05 mm. [Fischer and Mildbrand, 1924]. The monomeric form thus obtained was washed with anhydrous alcohol-ether mixture (1 : 1) to remove traces of glycerol and dried over phosphorus pentoxide; m.p. 72–80° (uncorr.).

Table VI shows the results of the estimations.

Table VI.

1 cc. $N/10$ I $\equiv$ 4.5 mg. dihydroxyacetone.

Mg. dihydroxyacetone		% yield
Used	Found	
6.00	5.79	97
3.00	2.95	98
1.50	1.47	98
0.751	0.755	100
0.375	0.380	101

¹ We wish to express our thanks to Prof. A. J. Kluyver of Delft for kindly supplying us with a culture of *Acetobacter suboxydans*.

(4) *dl-Glyceraldehyde*.

This substance was prepared from glycerol by the synthesis of Wohl [1898]. The details described by Witzemann [1914] were followed, except for the preparation of β -chloropropionacetal, in which the conditions of Reeves [1927], which give a superior yield, were employed; m.p. 139–40° (uncorr.). The solutions were allowed to stand in the presence of toluene and a trace of sulphuric acid for at least 48 hours before estimation, in order that the compound should pass over to the monomeric form. Table VII shows the results of the bisulphite titrations.

Table VII.

1 cc. <i>N</i> /10 I $\equiv$ 4.5 mg. glyceraldehyde.		
Mg. glyceraldehyde		
Used	Found	% yield
5.86	5.66	98
5.00	4.89	98
2.93	2.78	95
1.47	1.48	101
0.733	0.726	99
0.365	0.354	97

V. DETERMINATION OF THE CONSTANTS *K* AND *F* AND EXAMPLES OF THE ESTIMATION OF MIXTURES OF PYRUVIC ACID WITH METHYLGLYOXAL OR ACETALDEHYDE.

The determinations of the constant *K* with quantities of the alkali-labile carbonyl compounds varying between 6.0 and 0.2 mg. in each case are summarised in Table VIII.

Table VIII.

		Mean
Acetaldehyde	0.015, 0.007, 0.006, 0.006, 0.006, 0.006	0.008
Glyceraldehyde	0.13, 0.060, 0.030, 0.020, 0.020	0.05
Dihydroxyacetone	0.17, 0.12, 0.17, 0.15, 0.13	0.15
Methylglyoxal	0.014, 0.019, 0.009, 0.031, 0.010, 0.009, 0.010, 0.011, 0.042	0.014
Acetoacetic acid	0.190, 0.068, 0.048, 0.025, 0.029	0.07

The experimental error in these determinations is large owing to the small titrations after the alkali treatment. This however does not greatly affect the accuracy of the estimation, since *f* is small compared with *F*.

The figures for acetoacetic acid are based on the initial concentration determined by the Van Slyke method (see section VII).

The estimations of the percentage of pyruvic acid remaining after heating with alkali are somewhat inconsistent. We have been unable to find any explanation of this inconsistency. Triplicate titrations of samples of the same solution after heating with alkali show considerable variations.

Table IX gives examples of the results obtained.

Table IX.

Mg. pyruvic acid (in 5 cc.)	<i>F</i>		
6.0	0.914	0.905	0.992
2.8	0.945	0.960	0.755
1.4	0.869	0.903	0.949
0.7	0.897	0.955	0.940
0.35	0.906	0.926	0.104

The mean of a very large number of determinations gives $F = 0.93$.

The values of F obtained for hydroxymaleic acid are shown in Table X. The initial concentration of hydroxymaleic acid is based on the weight taken and not on the bisulphite titration (see section VII).

Table X.

Mg. hydroxymaleic acid (in 5 cc.)	<i>F</i>
7.93	0.82
3.97	0.87
1.98	0.81
0.99	0.91
0.50	0.93

Table XI shows the results obtained in the estimation of mixtures of pyruvic acid with methylglyoxal and acetaldehyde.

Table XI.

Mg. used			Titrations expressed in terms of cc. <i>N</i> /10 I	Mg. found		
Pyruvic acid	Methyl- glyoxal	Acetal- dehyde		Pyruvic acid	Methyl- glyoxal	Acetal- dehyde
1.64	—	0.63	$T_1 = 0.661$ $T_2 = 0.357$	1.67	—	0.62
1.64	—	1.27	$T_1 = 0.911$ $T_2 = 0.345$	1.61	—	1.19
1.64	0.96	—	$T_1 = 0.712$ $T_2 = 0.341$	1.59	1.00	—
1.64	1.91	—	$T_1 = 1.039$ $T_2 = 0.346$	1.64	1.92	—

VI. EXAMPLES OF THE RECOVERY OF ALDEHYDES AND KETONES ADDED TO TISSUES.

Technique.

10.0 g. quantities of the fresh rat tissue were finely chopped. An aliquot of a standard solution of the aldehyde or ketone under test was added, and after about 30 seconds' stirring, 5 cc. of 50 % trichloroacetic acid were added. The mixture was ground for a few minutes with sand and squeezed through muslin. The residue was extracted three or four more times by grinding with small quantities of water and again squeezing through muslin. The filtrate was where necessary (*viz.* in the methylglyoxal estimation) adjusted to about p_H 2 by the addition of *N* NaOH, made up to 100 cc. and re-filtered. 5 cc. aliquots

were then estimated in the usual manner. Allowance is made in each case for a tissue blank determined at the same time.

Table XII summarises the results.

Table XII.

Tissue	Substance added	Quantity added (mg.)	Quantity found	% recovery
Muscle	Pyruvic acid	66.7	64.8	97.1
	"	7.22	6.96	96.4
Liver	Methylglyoxal	44.1	38.8	88.0
"	"	8.75	5.56	63.6
Muscle	"	18.4	13.5	73.4
"	Acetaldehyde	12.4	4.34	35.0
"	Glyceraldehyde	44.6	44.2	99.1
Liver	"	44.6	43.7	98.0
"	Dihydroxyacetone	70.0	69.0	98.6
"	"	7.61	7.28	95.6

Tissue blanks.

10.0 g. rat-liver $\equiv$ 0.915, 0.474, 0.332 cc. *N*/10 *I*
 ,, muscle $\equiv$ 0.336, 0.457, 0.161 ,,

Considering the experimental difficulty of carrying out a quantitative extraction of the tissues and at the same time keeping the volume of the extract as small as possible, it may be assumed that the recovery of pyruvic acid and of the trioses is practically quantitative.

There are several indications in the literature that dilute solutions of methylglyoxal react in the cold with substances likely to be present in animal tissues. Thus Neuberg and Kobel [1927] showed that this keto-aldehyde reacted with amino-acids forming "melanin" together with CO_2 , NH_3 and acetaldehyde. Müller and Pechmann [1889] and also Pinner [1902; 1905] showed that phenylglyoxal readily condensed with ammonia. The reaction of phenylglyoxal with a constituent or constituents of the pancreas was also studied by Giršavičius [1930].

The failure to effect a quantitative recovery of acetaldehyde from muscle may be connected with the observation of Fabre [1925] that while a deproteinised blood-filtrate yields about 0.77 mg. of acetaldehyde per litre when this substance is distilled from the liquid in a current of nitrogen, the same filtrate only yields 0.48 mg. per litre when allowed to stand in the same closed air-space as the Nessler's reagent used for estimating it.

The tissue blanks observed with rat-muscle may be accounted for, at least in part, by the presence of pyruvic acid and methylglyoxal in the filtrates obtained under these conditions [Case and Cook, 1931]. We have made repeated attempts to isolate and identify the substance or substances responsible for the bisulphite-binding capacity of trichloroacetic acid extracts of ox-liver without success. We are at present only able to report that pyruvic acid is not amongst them.

VII. THE BEHAVIOUR OF β -KETONIC ACIDS AND ACETONE.

An attempt to apply the method to acetoacetic and oxaloacetic acids has shown that these substances cannot be estimated with any great accuracy. Our observations seem however to be worth recording, since they may be of use in a qualitative or semiquantitative manner. The reason for the inapplicability of the method to these substances is twofold. In the first place the bisulphite compounds appear to be sufficiently unstable in acid solution to render the first end-point ill-defined. Secondly a slow formation of iodoform occurs when the solutions of these substances stand in the presence of iodine and sodium bicarbonate. This renders the second end-point ill-defined. We record in Tables XIII and XIV the results of a series of determinations, taking as the end-points a blue coloration which lasted for about 15 seconds.

Table XIII.

Mg. acetoacetic acid		% of theory*
Used	Found	
7.11	6.16	86.6
3.56	3.09	86.7
1.78	1.54	86.5
0.889	0.778	87.4
0.444	0.397	89.4

* Calculated on the basis of 1 cc. $N/10$ I $\equiv$ 5.1 mg. acetoacetic acid.

Table XIV.

Mg. hydroxymaleic acid		% of theory*
Used	Found	
7.93	6.51	82.2
3.97	3.28	82.6
1.98	1.62	81.8
0.991	0.892	90.4
0.496	0.427	86.1

* Calculated on the basis of 1 cc. $N/10$ I $\equiv$ 6.6 mg. oxaloacetic acid.

The sodium acetoacetate solution was prepared free from acetone and ethyl acetoacetate by the method of Shaffer [1921] and standardised by the method of Van Slyke [1917]. The bisulphite titrations and Van Slyke determinations were both made within an hour of the removal of the solution from aeration flask (aeration time was 20 hours).

The oxaloacetic acid (hydroxymaleic acid) was prepared by the method of Simon [1903] from ethyl oxaloacetate and recrystallised from ethyl acetate by the addition of benzene. The substance softened at 145° and decomposed at 156° . The purity was checked by titration with alkali.

30.38 mg. required 4.58 cc. $N/10$ NaOH (phenolphthalein)

Calculated titre = 4.60 ,, ,,

Only freshly prepared solutions were used.

Acetone cannot be estimated owing to the instability of the bisulphite compound in solution.

VIII. INTERFERING SUBSTANCES.

Reduced glutathione, proteins, glycogen and urea do not interfere with the titration. After heating with alkali however, reduced glutathione solutions have a bisulphite-binding capacity which is probably due to the presence of pyruvic acid which is known to occur amongst the alkaline decomposition products of cystine [see *e.g.* Friedmann, 1903]. For example, 6.85 mg. of glutathione, after treating with alkali, required 0.0385 cc. *N*/10 I.

Unsaturated substances interfere to a small extent in relatively high concentrations. Thus

50 mg. of maleic acid	≡ 0.070 cc. <i>N</i> /10 I
50 „ fumaric acid	≡ 0.017 „
50 „ cinnamic acid	≡ 0.026 „

Bile has a slight bisulphite-binding capacity; *e.g.* 5 cc. of fresh ox-bile took 0.070 cc. *N*/10 I.

Commercial yeast nucleic acid gives a small titre. Thus 50 mg. took 0.022 cc. *N*/10 I.

The behaviour of glucose is remarkable. Neutral solutions of glucose (Kahlbaum) yield a slight titre. 5 cc. aliquots containing the below-mentioned amounts of glucose took the amounts of iodine indicated.

Mg. glucose	cc. <i>N</i> /10 I
460	0.15
50	0.05
10	0.01

The end-points are very ill-defined. After heating with alkali the titre is not sensibly increased. Fructose behaves similarly.

In the presence of ammonium salts or amino-acids, however, the titre of glucose is greatly increased. This is only the case if the solution is approximately neutral prior to the addition of the bisulphite. The presence of an amount of acid or base, small compared with the amount of acid represented by the bisulphite, reduces the titre to a value of the same order as it has in the absence of ammonium salts or amino-acids. For example 10 mg. of glucose in the presence of 5 mg. of ammonium chloride have a titre of the order of 0.7 cc. *N*/10 I. In the presence of *N*/100 HCl or *N*/100 NaOH this titre is reduced to 0.01 cc.

It is hoped to publish an investigation of this remarkable effect in a later communication.

SUMMARY.

1. A method of estimating pyruvic acid and methylglyoxal depending on the capacity of these substances to form compounds with bisulphite is described. The minimum amount of these substances to which the method is sensitive is 0.2 mg. of either substance in 5 cc.

2. From the fact that methylglyoxal and other aldehydes and ketones are destroyed by heating in alkaline solution while pyruvic acid and certain other ketones are not, a method has been devised by means of which the components of a mixture of one of the former with one of the latter may be determined.

3. The method is extended to the determination of glyceraldehyde, dihydroxyacetone and acetaldehyde.

4. The effect of compounds containing an ethylene linkage or a sulphydryl group has been studied. In general these compounds do not affect the estimation.

5. The methods of preparation of the carbonyl compounds studied are indicated and the question of some polymerides of pyruvic acid is discussed.

6. The method for the recovery and estimation of some of the above compounds in the presence of animal tissues is described.

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