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The Determination of Creatinine in Plasma or Serum, and in Urine; a Critical Examination

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Many different methods have been proposed for the determination of creatinine in plasma, serum and urine, and most of these have been based on the colour reaction which takes place between creatinine and sodium picrate in an alkaline medium (Jaffé, 1886). On comparison, however, these different analytical procedures have been found to give different results, particularly when applied to plasma or serum.

The Jaffé reaction is not specific for creatinine and it is generally acknowledged that there are present in plasma and serum, and, to a smaller extent, in urine, one or more unknown substances which also give an orange colour with alkaline picrate. Methods which have been claimed to be more specific towards creatinine include a nephelometric method (Barclay & Kenney, 1947) and various procedures employing 3:5-dinitrobenzoic acid in place of picric acid (Langley & Evans, 1936; Benedict & Behre, 1936; Bolliger, 1936). In addition, other methods have been devised to increase the specificity of the Jaffé reaction, and these involve the use of creatinine-destroying bacteria (Dubos & Miller, 1937), adsorption techniques (Brod & Kotátko, 1949; Hare, 1950; Haugen & Blegen, 1953) and destruction of interfering substances with ceric sulphate (Kostir & Rabek, 1950; Kostir & Sonka, 1952).

We have attempted to explain some of the differences in the results of creatinine determinations obtained with different methods by examining the factors which influence the determination of creatinine in plasma, serum and urine. Attention was confined to the methods based on the Jaffé reaction and to the procedures which have been claimed to increase the specificity of the reaction since methods based on other reactions did not appear to offer any definite advantages.

MATERIALS

Creatinine. Standard solutions were prepared daily by dilution of a stock solution containing 400 mg. creatinine/100 ml. of 0·1 N-HCl. The stock solution was stored at 0° and was never kept for longer than 4 weeks.

Picric acid. An aqueous solution, saturated at room temperature (18–20°), was prepared from picric acid (A.R.) which had been recrystallized twice from water and satisfied the criteria of purity proposed by Folin & Doisy (1917). The solution was kept in dark bottles and was made up at intervals of a few days.

Other reagents. Sodium hydroxide, (A.R.) 2·5 N. Sodium tungstate, (A.R.) a 10% (w/v) solution of Na_2WO_4, $2H_2O$. Sulphuric acid, (A.R.) 0·66 N. Phosphate buffer, 1 M, pH 7·0 (Green, 1933). Oxalic acid, (A.R.) saturated aqueous solution. Lloyd's reagent (hydrated aluminium silicate), Hartmann-Leddon Co., U.S.A.

NC-bacteria. A suspension of these bacteria (sometimes called *Corynebacterium ureafaciens*) was prepared according to the method of Miller, Allinson & Baker (1939).

Plasma, serum and urine were obtained from normal subjects; the plasma was heparinized.

A Unicam SP. 600 Spectrophotometer was used to measure optical densities.

METHODS

Jaffé reaction. This was carried out, unless otherwise stated, according to the method of Folin & Wu (1919). To 4·0 ml. of the solution containing creatinine were added 2·0 ml. of alkaline picrate solution and the colour was allowed to develop for 20 min. in a water bath at 20±0·2°. The alkaline picrate solution was made up immediately before use by adding 1 vol. of sodium hydroxide to 5 vol. of picric acid. The optical density of the developed colour was measured at a wavelength of 520 mμ. using water as reference optical density. For determinations in plasma or serum filtrates or in diluted urines, two standard solutions were included with each batch. All results were expressed in terms of creatinine.

Enzymic destruction of creatinine using the NC-bacteria. The method used was that described by Miller & Dubos (1937).

Adsorption of creatinine on Lloyd's reagent. The method used was that described by Haugen & Blegen (1953). Standard creatinine solutions were included with each batch and the temperature of the final coloured solutions was adjusted to 20° before measurement of the optical density.

Precipitation of plasma or serum proteins. Method I: to serum (2 vol.) were added water (6 vol.), sodium tungstate (1 vol.) and sulphuric acid (1 vol.) (Miller & Dubos, 1937). This gave a filtrate which, in this paper, will be termed 'neutral' filtrate, and is equivalent to plasma or serum diluted fivefold. Method II: to serum (2 vol.) were added water (3 vol.), sodium tungstate (1 vol.) and sulphuric acid (2 vol.) (Brod & Sirota, 1948). This gave a filtrate which we shall term 'acid' filtrate, and is equivalent to plasma or serum diluted fourfold.

RESULTS AND DISCUSSION

The Jaffé reaction

Light absorption by alkaline creatinine picrate. The absorption spectrum of alkaline creatinine picrate shows maximum absorption in the region of 490 mμ. (Fig. 1), but at wavelengths below 500 mμ. the optical density of alkaline picrate (i.e. the reagent blank) is also high (Fig. 1). Consequently filters having maximum transmission at wavelengths above 500 mμ. have generally been used. When measured with absorptiometers employing filters or diffraction gratings which provide light with a relatively broad waveband, the developed colour is reported not to obey Beer's Law (Bonsnes & Taussky, 1945; Hawk, Oser & Summerson, 1947; Lauson, 1951; Schoch & Camara, 1952; Hervey, 1953), but with instruments providing light with a narrow waveband Beer's Law is reported to be obeyed over the required concentration range (Borsook, 1935; Borsook & Dubnoff, 1940; Hervey, 1953). It has also been reported, however, that even with monochromatic light Beer's Law is not obeyed at all wavelengths (Garner, 1952).

We have confirmed the findings of Garner. The optical density of alkaline creatinine picrate was measured at wavelengths from 480 to 520 mμ. and plotted against the concentration of creatinine (Fig. 2). As the wavelength increased the relationship between the optical density and the concentration became increasingly more nearly linear, although the optical density itself decreased after a slight initial rise. At 520 mμ. the relationship was linear up to a concentration of 400 μg./100 ml. and at a concentration of 800 μg./100 ml. it deviated from linearity by only 2%. For this reason the wavelength of 520 mμ. was used in this investigation.

Picric acid. The optical density of alkaline creatinine picrate is independent of the picric acid concentration above a certain value, which is exceeded in all the methods which have been proposed (Bonsnes & Taussky, 1945).

Early workers noticed that picric acid which had been exposed to light (Hunter & Campbell, 1916), to high temperature (Wilson & Plass, 1917), or, in some instances, merely obtained commercially (Folin & Doisy, 1917),

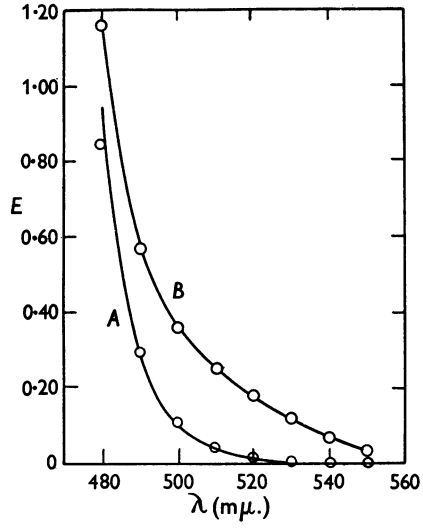

Fig. 1. Absorption spectra. *A*, alkaline picrate reagent diluted 1:2 with water (reagent blank). *B*, alkaline creatinine picrate and alkaline picrate (4 ml. creatinine (200 μg./100 ml.) + 2 ml. alkaline picrate). Colour developed for 20 min. at 20°. 10 mm. cells.

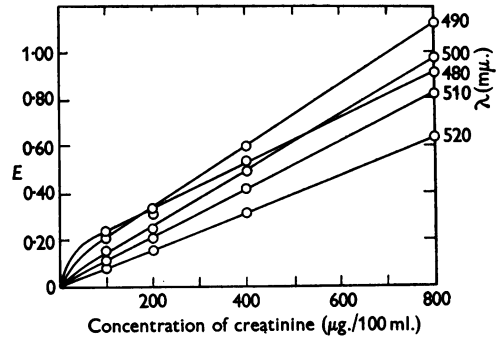

Fig. 2. Optical density of alkaline creatinine picrate at various wavelengths. 4 ml. creatinine solution and 2 ml. alkaline picrate; colour developed for 20 min. at 20°. 20 mm. cells.

contained material which on the addition of sodium hydroxide produced a red colour similar to that produced by creatinine. To eliminate this source of error various methods have been proposed for the purification of picric acid (Folin & Doisy, 1917; Benedict, 1929; King, 1951).

We have examined the effect of exposing picric acid solution to daylight, with intermittent sunlight, for 4 weeks. The optical density of the colours produced when creatinine solutions were added to alkaline picrate made from this picric acid were compared with those produced with alkaline picrate made from freshly prepared picric acid. The optical density of the alkaline picrate made with 'light-exposed' picric acid was nearly 4 times that of the alkaline picrate made with freshly prepared picric acid yet the optical density of alkaline creatinine picrate was unaltered. Hence, with a modern sensitive spectrophotometer the purification of the picric acid and the avoidance of exposure to light is perhaps less important than when less sensitive instruments are employed.

Sodium hydroxide and the effect of pH. The optical densities of alkaline picrate and of alkaline creatinine picrate depend on the pH of the final solution which, in turn, depends on the concentration of the sodium hydroxide and on the acidity of the original solutions containing creatinine. The optical density of alkaline picrate is reported to be a direct function of the pH of the final solution, while the optical density of alkaline creatinine picrate is an indirect function of the pH of the final solution (Bonsnes & Taussky, 1945; Roscoe, 1953).

We have examined this point by adding alkaline picrate to (*a*) sulphuric acid solutions of varying concentration and (*b*) creatinine solutions containing comparable quantities of sulphuric acid. On account of the strongly alkaline nature of the final mixture it was considered impracticable to measure its pH directly, and the optical densities have therefore been related to the titratable acidity of the fluids to which the alkaline picrate was added, since it was this which determined the pH of the final solutions.

Table 1. *Effect of acidity of test solution on the optical density of alkaline picrate and alkaline creatinine picrate*

Alkaline picrate (2 ml.) was added to 4 ml. of each of the following: (*a*) water; (*b*) creatinine solution, 200 μg./100 ml.; (*c*) creatinine solution, 400 μg./100 ml.—each containing various comparable quantities of 0·66 N-H_2SO_4. Colour developed for 20 min. at 20°. Optical densities measured at 520 mμ., 20 mm. cells.

Titratable acidity of original soln. (ml. 1·0 N-NaOH/100 ml.)	Optical density (% of value in water)		
	a	*b*	*c*
0·0	100	100	100
4·2	88	102·5	102
8·3	76	105	105·5
12·5	62	109	107

The optical density of alkaline picrate decreased with increasing acidity of the sulphuric acid solution added (Table 1), and it follows, therefore, that the reagent blank applied to an acid medium is less than when applied to water. On the other hand, the optical density of alkaline creatinine picrate increased as the acidity of the creatinine solution was increased although the relative change was much less. The optical density of alkaline creatinine picrate is thus greater when prepared from an acid medium than from a neutral medium.

Effect of temperature. Many workers have noted that variations in the temperature, at which the reaction is carried out, affect the determination of creatinine.

We have confirmed that an increase in temperature causes an increase in the optical densities of alkaline picrate and of alkaline creatinine picrate. Furthermore, we have found that this effect is reversible. Taking as a basis for comparison the optical density when the colour was developed and measured at 20°, development and measurement at 15° gave figures about 5% lower for both alkaline picrate and alkaline creatinine picrate; development and measurement at 25° gave figures about 25% higher for alkaline picrate and 10% higher for alkaline creatinine picrate. Further analysis showed that the temperature of development was immaterial within the range 15–25° and that the differences in optical density were due solely to the temperature at the time of measurement. The temperature, and therefore the optical density, of the alkaline picrate in the reagent blank, increases when the solution is repeatedly exposed to the heating effect of the light beam of the absorptiometer (Haugen, 1953). Consequently water or air should be used as a reference optical density.

The reversibility of the effect of temperature becomes important when creatinine is determined using Lloyd's reagent according to the method of Haugen & Blegen (1953), since it is not always possible to keep the solution containing the developing colour at a constant temperature, e.g. during centrifuging, and it may, therefore, be necessary to adjust the temperature of the solutions immediately before determining the optical densities.

Colour development. After the addition of alkaline picrate to a pure creatinine solution the optical density reaches a maximum within a short time, 10–20 min., and remains constant for several hours (Hunter & Campbell, 1916; Bonsnes & Taussky, 1945; Hare, 1950; Haugen, 1953). When creatinine is originally present in an acid medium, a longer time is required for the development of maximum optical density (Roscoe, 1953). Diluted urine behaves in a manner similar to pure creatinine solutions. In plasma or serum filtrates, however, a colour is produced which does not reach a maximum optical density for several hours. This is believed to be due to the presence of one or more unknown

substances, termed 'non-creatinine chromogen' (Hunter & Campbell, 1916; Hayman, Johnson & Bender, 1935; Hare, 1950; Haugen, 1953).

We have examined the rate of colour development in various media (Fig. 3). In pure creatinine solution the optical density reached a maximum 16 min. after the addition of alkaline picrate, and remained constant for longer than 1 hr. In diluted urine, and in the eluates obtained from Lloyd's reagent which had been shaken with pure creatinine solution (or serum filtrate, or diluted urine), the rate of colour development was similar to that in pure creatinine solution and the optical density likewise remained at its maximum value for 1 hr. In untreated filtrates, however, and in filtrates which had been treated with the NC-bacteria, colour development was initially rapid and then continued to increase at a slower rate so that the maximum optical density was not reached in 1 hr.

Effect of other substances on the Jaffé reaction. The optical density of alkaline creatinine picrate is variably affected by the presence of other substances. Richter (1944) and Lauson (1951) reported that glucose, which is itself chromogenic, depresses the optical density of alkaline creatinine picrate and we have confirmed this. Other substances are reported to increase the optical density of alkaline creatinine picrate.

That such an effect might occur in plasma filtrate was first suggested by Gaebler & Keltch (1928) and, indeed, Lauson (1951) demonstrated that when alkaline picrate is added to a mixture of creatinine and the non-creatinine chromogen from plasma, the optical density of the colour developed by the mixture was less than the sum of the optical densities of the two colours developed separately.

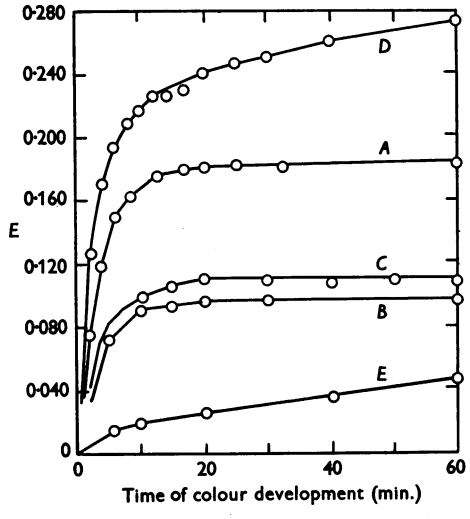

Fig. 3. Rate of colour development on the addition of alkaline picrate to various solutions. 4 ml. test solution and 2 ml. alkaline picrate; colour developed at 20°; wavelength 520 mμ. 20 mm. cells. (*A*) creatinine in water, 200 μg./100 ml., (*B*) diluted urine, (*C*) eluate from Lloyd's reagent shaken with 'neutral' serum filtrate, (*D*) 'neutral' serum filtrate, (*E*) 'neutral' serum filtrate after incubation with NC-bacteria.

Methods for increasing the specificity of the Jaffé reaction towards creatinine

These are based on three principles: (1) The destruction of interfering substances with ceric sulphate (Kostir & Rabek, 1950; Kostir & Sonka, 1952). (2) The destruction of creatinine by bacteria (Dubos & Miller, 1937). (3) The adsorption of creatinine on Lloyd's reagent (Gaebler, 1930).

Ceric sulphate method. Kostir & Rabek (1950) and Kostir & Sonka (1952) proposed the treatment of serum filtrates with ceric sulphate to destroy a non-creatinine chromogen which they found to constitute about half of the total chromogen present and which they believed to be pyruvic acid.

We have examined the effect of ceric sulphate on the chromogen content of serum filtrates using a slightly modified method. No significant difference was found between the total chromogen contents of serum filtrates before and after treatment with ceric sulphate. Further, the colour obtained on the addition of alkaline picrate to solutions of pyruvic acid was such that the small amount of pyruvic acid which is believed to be present in serum could account for only a small fraction of the non-creatinine chromogen content.

For these reasons no further studies were made with this method.

Determination of creatinine using the NC-bacteria. This method has been extensively used by Miller & Dubos (1937), Miller *et al.* (1939), Allinson (1945) and Miller & Miller (1951), who have justified its use on the following evidence: (1) The NC-bacteria quantitatively destroy creatinine added to plasma or serum filtrates or to diluted urine. (2) Creatinine is the only known chromogen which, being present in plasma filtrates and destroyed by the NC-bacteria, could account for the disappearance of chromogen in filtrates treated with these bacteria.

We have examined the effect of the NC-bacteria on the chromogen in serum filtrates, prepared by methods I and II, and in diluted urines, before and after the addition of creatinine. Not all the chromogen originally present was destroyed by the bacteria, whereas creatinine added in concentrations from 100 to 250 μg./100 ml. was quantitatively destroyed (Table 2).

The effect on the reaction of increasing the number of bacteria was examined by doubling the volume of the bacterial suspension added to a 'neutral' filtrate, removing samples of the incubating mixture at intervals, stopping the reaction by heating rapidly to 100° and determining the chromogen present. Although chromogen disappeared from the filtrate more rapidly, the final amount of chromogen remaining was unaltered.

This finding suggests that the bacteria do not produce any appreciable amount of the non-creatinine chromogen present in 'neutral' tungstate filtrate. However, there still remains the possibility that one or more unknown chromogens are also destroyed by the bacteria.

Adsorption method using Lloyd's reagent. This has been used, with different modifications, by Gaebler (1930), Borsook (1935), Danielson (1936), Brod & Kotátko (1949), Hare (1950) and Haugen & Blegen (1953), who have justified its use on the following evidence: (1) Creatinine added to plasma or serum filtrates and to diluted urines is quantitatively adsorbed on Lloyd's reagent and subsequently can be eluted quantitatively. (2) The rate of colour development on the addition of alkaline picrate to the eluate obtained from Lloyd's reagent after being shaken with serum filtrate or diluted urine is identical with that in pure creatinine solutions when similar conditions of time, temperature and pH are imposed. (3) Lloyd's reagent does not adsorb any of the chromogen occurring in filtrates after these have been treated with the NC-bacteria (Miller & Miller, 1951).

We have confirmed that Lloyd's reagent quantitatively adsorbs creatinine added to serum filtrates and to diluted urine (Table 4) and we have also confirmed that the colour development in the eluate is similar to that in solutions of pure creatinine (Fig. 3).

The whole of this evidence does not exclude the possibility that Lloyd's reagent adsorbs other chromogens, or the possibility that some creatinine is formed from a precursor during the process of adsorption and elution, as was suggested by Gaebler (1930). In addition, it must be noted that not all samples of Lloyd's reagent have equal ability to adsorb creatinine (Haugen & Blegen, 1953). Nevertheless, a satisfactory sample of this reagent is superior as an adsorption reagent to kaolin, synthetic magnesium trisilicate or ion-exchange resins, all of which suffer from the disadvantage of inefficient adsorption or high reagent blanks.

Table 2. *Destruction by NC-bacteria of creatinine added to serum filtrates and to diluted urine*

Serum filtrate or diluted urine (5·0 ml.), phosphate buffer (0·2 ml.), bacterial suspension (0·5 ml.); incubated 37° C. for 60 min. The concentration of added creatinine ranged from 100–250 μg./100 ml. All values recorded as creatinine.

Medium	Initial total chromogen (μg./100 ml.)	Non-creatinine chromogen (μg./100 ml.)	Non-creatinine chromogen after addition of creatinine (μg./100 ml.)
Serum filtrate, method I	159	36	36
	168	28	27
	206	36	32
	155	26	26
	156	30	26
	187	51	37
	175	37	32
	160	33	35
Serum filtrate, method II (adjusted to pH 7·0)	247	86	72
	188	42	47
	216	60	50
Diluted urine	222	2	2
	184	2	8
	135	10	16
	202	5	3
	142	9	10
	205	9	8
	143	9	9

Recovery of creatinine added to plasma or serum or to protein-free filtrates prepared from these

Protein precipitation. The various procedures which have been used for the preparation of protein-free filtrates of plasma or serum have been reported to give different values for the recovery of added creatinine. These procedures may be divided conveniently into two groups: those which provide a 'neutral' filtrate (pH > 2·5; very low titratable acidity) and those which provide an 'acid' filtrate (pH < 2·5; considerable titratable acidity). Filtrates obtained by using Na_2WO_4 (10% w/v) and H_2SO_4 (0·66N) in equal volumes (e.g. Method I), by using $Cd(OH)_2$, or by using ultrafiltration belong to the first group. Filtrates obtained by using Na_2WO_4 and larger proportions of H_2SO_4 (e.g. method II), or by using trichloroacetic acid belong to the second group.

When mixed, equal volumes of Na_2WO_4 (10%, w/v) and H_2SO_4 (0·66N) give an acid solution. The excess H_2SO_4 is partly neutralized by the proteins if plasma or serum is present. The actual pH of the filtrate is determined by the relative amounts of Na_2WO_4, H_2SO_4, serum and water, and for any given proportion of these reagents the pH of the filtrate also depends on the protein concentration in the serum.

The mean pH values of filtrates prepared from five sera using various procedures are given in Table 3.

Recoveries of creatinine previously reported. The recovery data of previous workers who have used tungstic acid as a protein precipitant are summarized in Table 3. Using trichloroacetic acid and Lloyd's reagent, Hare (1950) obtained recoveries of creatinine added to serum in the range 98–100%, whilst Mandel & Jones (1953) with the same procedure obtained a mean recovery of 102%. Using $Cd(OH)_2$ and Lloyd's reagent the latter workers obtained a mean recovery of 93%.

From these data it is evident that with some methods of preparing protein-free filtrates the recovery of creatinine added to plasma or serum is considerably less than the recovery of creatinine added to protein-free filtrates. Camara, Arn, Reimer & Newburgh (1951) suggested that this results from the retention of creatinine on the precipitated protein. Lauson (1951) examined this pheno-

Table 3. *Recovery of creatinine using various types of tungstate filtrates*

Abbreviations for methods of creatinine determination. Chr., total chromogen; L.R., Lloyd's reagent; NC-B, NC-bacteria.

Plasma or serum (vol.)	Water (vol.)	Sodium tungstate (10%, w/v) (vol.)	Sulphuric acid (0·66 N) (vol.)	Mean pH (present authors)	Method of creatinine determination	Mean recovery of creatinine (various authors): From plasma or serum (%)	From filtrates (%)	References
1·0 (method II)	1·5	0·5	1·0	1·58	Chr.	100	—	(1)
					Chr.	100	98	(3)
					Chr.	99	—	(4)
					L.R.	100	—	(4)
					Chr.	100	—	(5)
					Chr.	100	104	(6)
					L.R.	98	104	(6)
					NC-B	100	—	(6)
1·0	2·0	1·0	1·0	2·52	Chr.	78	99	(3)
					Chr.	88	—	(4)
					L.R.	81	—	(4)
1·0	7·0	1·0	1·0	2·62	Chr.	88	100	(2)
					Chr.	85	96	(3)
					Chr.	90	—	(4)
					L.R.	91	—	(4)
1·0	2·0	0·5	0·5	4·00	Chr.	90	—	(1)
					Chr.	78	95	(2)
					Chr.	86	98	(5)
1·0 (method I)	3·0	0·5	0·5	4·06	Chr.	89	100	(6)
					L.R.	94	100	(6)
					NC-B	91	100	(6)
					Chr.	97	—	(4)
					L.R.	94	—	(4)
1·0	8·0	0·5	0·5	4·16	Chr.	94	100	(3)

References: (1) Brod & Sirota (1948), (2) Camara *et al.* (1951), (3) Haugen (1953), (4) Mandel & Jones (1953), (5) Roscoe (1953), (6) Present authors.

menon further, comparing directly the recovery of creatinine added to plasma with the recovery from 'neutral' tungstate filtrates of the same plasma and found the recovery from plasma was on the average 94% (90–98%) of that from the filtrates. He noted that the recovery from plasma varied with the plasma protein concentration, in that the recovery from plasma with a low protein concentration was less than that from a plasma with a high protein concentration. This finding is apparently contrary to the conclusions of Camara *et al.* and suggests that any adsorption of creatinine on the precipitated protein is not dependent solely on the amount of the precipitate present.

Recoveries of creatinine obtained by the present authors. We have examined the recovery of creatinine added to serum and to serum filtrates, using in each case filtrates prepared by methods I ('neutral') and II ('acid'). Creatinine was determined as the total chromogen; using the NC-bacteria; and using Lloyd's reagent. The mean recoveries of creatinine added to serum by all methods at all levels of added creatinine were higher with 'acid' filtrates than with 'neutral' filtrates. The recovery of creatinine added to serum filtrates tended to be slightly higher with 'acid' filtrates (Table 4).

The effect of the pH of the filtrate on the recovery of creatinine added to serum or to filtrates was further examined by adding various amounts of sulphuric acid to the protein-precipitating mixture and determining the recovery of added creatinine. Minimum recovery from serum was obtained when filtrates with pH values between 3 and 4 were used (Fig. 4*A*). Recoveries of creatinine added to filtrates averaged 100% and were independent of pH except in filtrates with pH less than 2 in which recoveries tended to be slightly greater than 100% (Fig. 4*B*).

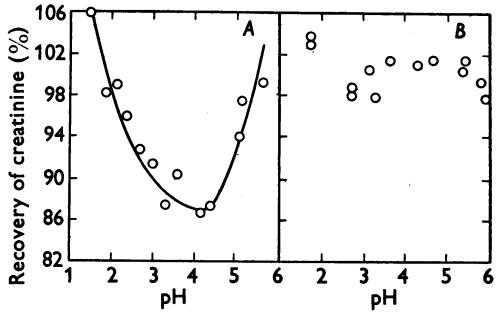

Fig. 4. Effect of pH of serum filtrate on the recovery of creatinine added to (*A*) serum, (*B*) serum filtrate.

Table 4. *Recovery of creatinine*

Mean recovery as percentage of added creatinine. Values ± are S.D. The no. of determinations is in parentheses. For small numbers of determinations, the range is shown.

		Recovery (%)		
	mg./100 ml.	Total chromogen	NC-bacteria	Lloyd's reagent
Creatinine added:				
to serum, using 'neutral' filtrates	1·0	88·8 ± 2·8 (14)	90·8 ± 3·7 (6)	93·9 ± 1·9 (6)
	10·0	91·5, 86–97 (4)	—	—
	100·0	92·0, 88–97 (4)	—	—
to serum, using 'acid' filtrates	1·0	99·7 ± 3·9 (12)	100·0 ± 6·8 (6)	97·6 ± 2·4 (6)
	10·0	96·7, 94–99 (4)	—	—
	100·0	96·3, 94–98 (4)	—	—
to 'neutral' filtrates	0·20–0·40	100·0, 95–102 (5)	—	99·9, 97–102 (7)
to 'acid' filtrates	0·20–0·40	103·8, 99–108 (6)	—	103·8, 100–108 (4)
to diluted urines	0·10	100·9, 97–110 (13)	101·4, 95–106 (7)	99·2, 96–105 (5)

When the mean recoveries of creatinine added to plasma or serum reported by various authors are arranged according to the pH of filtrates (as determined in the present investigation), there is the same tendency for the recovery to be minimal at a pH intermediate between that of filtrates prepared by methods I and II (Table 4).

Three explanations can be offered for the finding of higher recoveries of creatinine added to serum when 'acid' filtrates are used.

(1) Since the optical density of alkaline creatinine picrate is increased when the colour is developed in a medium originally acid, and the colours developed in 'acid' serum filtrates were compared with those developed from standard creatinine solutions in water, the increment in colour density (i.e. the apparent recovery) is greater the more acid the serum filtrate. In other words, the different recoveries given with 'acid' and 'neutral' filtrates are apparent and not real.

(2) Loss of creatinine may occur as a result of adsorption on the precipitated protein.

(3) Roscoe (1953) suggested that creatinine was modified in some way during the precipitation of protein so that the optical density of the alkaline creatinine picrate was reduced, claiming that recoveries from 'neutral' filtrates were increased if the filtrate was diluted before development of the colour.

We have not been able to confirm this finding of Roscoe and, furthermore, the recovery of large amounts (10 and 100 mg. creatinine/100 ml.) added to serum, which necessarily involves considerable dilution of the filtrates, still gave mean recoveries of only 92 % when 'neutral' filtrates were used.

It would seem likely, however, that both factors involved in the first and second explanations may contribute to the observed difference in recoveries. The first is undoubtedly responsible for the part of the increased recovery noted when creatinine is determined as total chromogen and is consistent with the observation that recovery from an 'acid' filtrate is frequently greater than 100 % (Table 4 and Fig. 4). However, the acidity of the filtrate cannot be solely responsible for the increased recoveries obtained since, with Lloyd's reagent, the colours in the eluates are all developed under the same conditions and still the 'acid' filtrates give better recoveries than the 'neutral' filtrates. We consider, therefore, that loss of creatinine by adsorption on the protein precipitate does occur, as was originally suggested by Camara *et al.* However, because of the difficulty of obtaining the protein precipitate sufficiently free from filtrate, it was not found possible to test this suggestion directly.

The direct dependence of the recovery of creatinine added to plasma on the protein concentration of the plasma, as reported by Lauson (1951), is consistent with our findings, for decrease in the plasma protein concentration causes a decrease in the pH of the filtrate and, provided this is still above the value giving a minimum recovery, there is decreased recovery (Fig. 4*A*).

In contrast to recoveries from plasma or serum, it is generally agreed that the recovery of creatinine added to normal urine is quantitative, and this is suggestive in relation to the possible immobilization of creatinine in serum as a precipitable protein complex. Our recoveries of creatinine added to diluted urine are given in Table 4.

Creatinine content of plasma or serum as determined by different methods

In addition to the factors already considered, the presence of non-creatinine chromogen must be taken into account, and relevant to this may be the method of preparation of protein-free filtrates.

Ferro-Luzzi (1934) and Ferro-Luzzi, Saladino & Santamama (1935) reported that zinc hydroxide filtrates of plasma contained less total chromogen than 'neutral' tungstate filtrates, which Danielson (1936) reported to contain essentially the same amount of chromogen as ultrafiltrates (We have confirmed this with both results

expressed as mg. creatinine/100 ml. of plasma). Smith, Finkelstein & Smith (1940) reported that picric acid filtrates contained less total chromogen than ferric carbonate filtrates. Brod & Sirota (1948) reported that 'neutral' and 'acid' tungstate filtrates contained the same amount of total chromogen, although the recoveries of creatinine added to plasma using these filtrates were 90 and 100% respectively. Camara *et al.* (1951) reported that 1 in 4 'neutral' tungstate filtrates contained 21% more total chromogen than 1 in 10 filtrates similarly prepared; the recoveries of creatinine added to plasma, however, were only 78 and 88% respectively.

Haugen (1953) determined the total chromogen content of sera, using different tungstate filtrates, and found the mean values obtained with three 'neutral' filtrates were practically the same as with 'acid' filtrates but these mean values bore no consistent relation to the different mean recoveries obtained for creatinine added to serum using the respective filtrates. Moreover, he found the rate of colour development differed with different filtrates and concluded that they contained different amounts of non-creatinine chromogen.

Determinations using the allegedly specific methods have shown, however, that varying amounts of non-creatinine chromogen are only partly responsible for differences in the total chromogen content (Miller & Miller, 1951; Mandel & Jones, 1953).

Comparative analyses by the present authors

We have made comparative determinations in 'neutral' and 'acid' filtrates of sera, determining 'creatinine' as total chromogen, with NC-bacteria and with Lloyd's reagent (Table 5). Since all the sera were not analysed by all three methods the figures in this table can be used only to compare 'acid' and 'neutral' filtrates. There was no difference between the mean value for total chromogen in 'acid' filtrates and in 'neutral' filtrates. Since, however, the optical density of alkaline picrate and alkaline creatinine picrate depend upon pH, the total chromogen contents of a further series of sera were determined, the 'acid' filtrates being adjusted to pH 7 before addition of alkaline picrate. The mean value for the total chromogen content using neutralized 'acid' filtrates was 5% (2–13%) higher than when 'neutral' filtrates were used.

Using the NC-bacteria, the difference between the chromogen content before and after incubation with the bacterial suspension was 17% lower in 'acid' filtrates, which had been adjusted to pH 7 with sodium hydroxide to obtain the correct pH for the bacterial activity, than in 'neutral' filtrates. In this series the total chromogen in the 'acid' filtrates was determined without prior neutralization and the figures should therefore be increased by 5% on the average. If this correction is made, however, the creatinine content of the 'acid' filtrates still appears to be significantly lower than that of the 'neutral' filtrates (mean corrected ratio 0·88).

Using Lloyd's reagent, the chromogen adsorbed on this reagent and eluted by the alkaline picrate was 5% higher in 'acid' filtrates than in 'neutral' filtrates, and this difference was statistically significant ($P<0·05$). This suggests that the total chromogen ratio (1·05) for 'acid' and 'neutral' filtrates is due to a difference in creatinine content and supports the idea of adsorption of creatinine on precipitated protein.

In certain sera all three techniques were applied to both 'acid' and 'neutral' filtrates. On comparing the results, it was evident the 'creatinine' content determined by the NC-bacteria or with Lloyd's reagent was always less than the total chromogen content (Table 6). Comparing the two 'specific' methods, it appeared that the mean 'creatinine' content obtained with the NC-bacteria in 'neutral' filtrates was 5% lower than that obtained with Lloyd's reagent, but this difference was not statistically significant. In 'acid' filtrates the mean value obtained with the NC-bacteria was 18% (corrected 13%) lower than that obtained with Lloyd's reagent. This difference, due to the apparent smaller 'creatinine' content of 'acid' filtrates as

Table 5. *'Creatinine' content of normal serum. Comparison of 'neutral' and 'acid' filtrates*

Results expressed as means ±S.D. No of observations in parentheses. These means can be used only to compare 'neutral' and 'acid' filtrates, since sera were not always analysed by all three methods.

Filtrate	Total chromogen (*a*) (mg./100 ml.)	NC-bacteria non-creatinine chromogen (*b*) (mg./100 ml.)	(*a*–*b*) (mg./100 ml.)	Lloyd's reagent (mg./100 ml.)
'Neutral'	0·886±0·173 (14)	0·185±0·057 (8)	0·715±0·116 (8)	0·716±0·082 (10)
'Acid'	0·874±0·189 (14)	0·267±0·071 (8)	0·601±0·154 (8)	0·756±0·086 (10)
Ratio: $\frac{\text{'Acid'}}{\text{'Neutral'}}$	1·02±0·019 (14)	0·695±0·073 (8)	0·835±0·124 (8)	1·05±0·052 (10)
	$P<0·5$	$P<0·01$	$P<0·05$	$P<0·05$

P = probability of mean ratio differing from 1·0 by chance alone.

determined with NC-bacteria and to the apparently larger 'creatinine' content obtained with Lloyd's reagent, was statistically significant.

The anomalous finding of a greater proportion of non-creatinine chromogen, determined by the NC-bacteria, in 'acid' filtrates than in 'neutral' filtrates, was investigated further by determining the chromogen content of 'neutral' and 'acid' filtrates prepared from plasma or serum which had been dialysed for 6 days against running tap-water. In some cases creatinine was added to the serum or plasma before dialysis. The chromogen content was determined as total chromogen, with the NC-bacteria and with Lloyd's reagent. Results are shown in Table 7.

The values for mean total chromogen content were slightly less than the mean non-creatinine chromogen normally present in serum as given by the difference between the value obtained with Lloyd's reagent and the total chromogen content. Using the NC-bacteria, the mean non-creatinine chromogen content was practically identical with that obtained with undialysed sera (Table 5).

Since the total chromogen values obtained in the dialysis residues from serum and plasma to which creatinine had been added were practically the same as those from plasma or serum without added creatinine, and since none of this chromogen was destroyed by the NC-bacteria it was concluded that none of the non-dialysable chromogen present in these filtrates was, in fact, creatinine.

The finding of identical amounts of total chromogen in 'neutral' and in unneutralized 'acid' filtrates is in agreement with the findings of Brod & Sirota (1948) and of Haugen (1953), although this is apparently inconsistent with the increased recoveries of creatinine obtained when 'acid' filtrates are used.

We suggest that this is due to the interaction of three factors: (1) the 'blank', i.e. the optical density due to alkaline picrate is really less when the latter is added to 'acid' filtrates than to creatinine standard solutions or to 'neutral' filtrates (Table 1); (2) a given amount of creatinine produces with alkaline picrate a greater optical density when present in an 'acid' than in a 'neutral' filtrate (Table 1); (3) an 'acid' filtrate from serum actually contains about 5% more creatinine than a 'neutral' filtrate of the same serum, as the recovery experiments indicate.

Table 6. *'Creatinine' content of normal serum. Comparison of methods*

Results expressed as mean ratio (±S.D.) of 'creatinine' content as determined by different methods. No. of observations in parentheses.

	Ratios		
	NC-bacteria / Total chromogen	Lloyd's reagent / Total chromogen	NC-bacteria / Lloyd's reagent
Filtrate			
'Neutral'	0·78±0·051 (10) $P<0·01$	0·81±0·039 (10) $P<0·01$	0·95±0·087 (12) $P<0·3$
'Acid'	0·67±0·126 (8) $P<0·01$	0·87±0·038 (10) $P<0·01$	0·82±0·097 (8) $P<0·05$

P = probability of mean ratio differing from 1·0 by chance alone.

Table 7. *Chromogen in dialysed serum or plasma*

Results in terms of creatinine/100 ml. dialysed serum or plasma.

		Chromogen (mg./100 ml.)					
		'Neutral' filtrate			'Acid' filtrate		
Material	Total protein (N × 6·25; g./100 ml.)	Total chromogen	NC-bacteria	Lloyd's reagent	Total chromogen	NC-bacteria	Lloyd's reagent
Serum A	3·1	0·14	—	0·08	0·14	—	0·04
Serum B	4·4	0·16	0·18	0·06	0·17	0·30	0·04
Serum C	5·1	0·16	—	0·00	0·10	—	0·00
Serum C + 10 mg./100 ml. creatinine	4·8	0·10	—	0·00	0·08	—	0·00
Serum C + 20 mg./100 ml. creatinine	5·0	0·16	—	0·00	0·20	—	0·00
Plasma D	4·5	0·09	0·15	0·05	0·13	0·25	0·06
Plasma D + 10 mg./100 ml. creatinine	4·4	0·13	0·20	0·06	0·16	0·23	0·05
Mean	—	0·13	0·18	0·04	0·14	0·26	0·03

When the effect of the acidity is removed either by neutralization of the 'acid' filtrates or by adsorption of the creatinine on Lloyd's reagent, the values for the total chromogen and 'creatinine' contents, respectively, are higher in the 'acid' filtrates, and this is consistent with the higher recoveries found using these filtrates.

The multiplicity of the factors involved makes it very difficult to draw definitive conclusions as to the technique which can be regarded as specific and accurate. It is clear that the NC-bacteria are capable of destroying creatinine completely and that Lloyd's reagent can adsorb creatinine quantitatively. It is also clear, from recovery experiments, that the 'acid' filtrate is preferable to the 'neutral'. On this basis it would seem that either of the specific techniques applied to an 'acid' serum filtrate should give reliable results. The two methods do, in fact, give significantly different results and this seems to be due chiefly to the anomalous behaviour of the NC-bacteria which show apparently greater quantities of non-creatinine chromogen in 'acid' filtrates from which the creatinine has been removed by the bacterial action and in 'acid' filtrates of dialysed serum from which the creatinine has already been removed. On the whole, therefore, it is fair to accept the Lloyd's reagent technique applied to 'acid' filtrates as giving the more accurate estimate of the true creatinine present. This technique has also the additional advantage of greater simplicity, and the reproducibility of results is very good. The standard deviation of determinations on twelve samples of one serum containing 0·85 mg./100 ml. creatinine was 1·5 %.

Comparison of chromogen contents of urine determined by different methods

The chromogen contents of twelve diluted urines were also examined using the three methods. The mean 'creatinine' content, determined with the NC-bacteria, was 229 μg./100 ml., i.e. 3 % lower than the mean total chromogen content (235 μg./100 ml.), the mean ratio of the respective values being 0·97; this difference was statistically significant. The mean 'creatinine' content determined with Lloyd's reagent was 223 μg./100 ml., 6 % lower than the total chromogen content, the mean ratio being 0·95; this difference was also statistically significant.

The mean creatinine content determined with Lloyd's reagent was 3 % lower than the mean creatinine content determined by the NC-bacteria, the mean ratio being 0·98. The difference between these results was not, however, statistically significant.

CONCLUSIONS

An examination of the Jaffé reaction has shown that the time and temperature of the colour development and measurement, and the pH of the medium must be carefully controlled. Using the alkaline picrate reagent of Folin & Wu the optical density of the colour obtained after 20 min. at 20° and measured at a wavelength of 520 mμ. is directly proportional to the amount of creatinine present, up to a concentration of 400 μg./100 ml.

The optical density of alkaline picrate and of alkaline creatinine picrate is dependent on the pH of the final solution. Since the pH of serum filtrates varies according to the method of preparation, it is necessary, for the accurate determination of 'creatinine' in these media, that the spectrophotometer be calibrated with standard solutions of creatinine of similar titratable acidity, or, more conveniently, that the serum filtrates be neutralized.

The recovery of creatinine added to plasma or serum is influenced by the pH at which the protein-free filtrate is prepared and is minimal (85–90 %) with filtrates having a pH of about 3·5. When the filtrate pH is below 2·0 recovery is quantitative. On the other hand, provided allowance is made for the effect of the acidity of the filtrate on the colour densities, the recovery of creatinine added to serum filtrates is independent of the pH and is always virtually 100 %. Similarly, recoveries of creatinine added to urine are independent of the method used and are also virtually 100 %. The importance of preparing the serum filtrate so that its pH is below 2·0 is thus evident; the incomplete recovery at higher pH levels is believed to be due to adsorption of creatinine by the precipitated protein.

When the Jaffé reaction is applied to the determination of creatinine in plasma, serum, or urine, allowance must be made for the presence of non-creatinine chromogens. Using the NC-bacteria or Lloyd's reagent the creatinine content of serum filtrates, or of diluted urines, is lower than the total chromogen content by a statistically significant amount. Neither of the two methods, however, is entirely satisfactory.

The adsorption method, using Lloyd's reagent, while improving the specificity, also eliminates the effect of the acidity of the filtrate and, possibly, the effect of other filtrate constituents, on the colour due to creatinine. However, experiments using dialysed plasma or serum suggest that a little non-creatinine chromogen present in filtrates can be adsorbed on Lloyd's reagent.

The use of the NC-bacteria also improves the specificity, but the NC-bacteria can destroy possible interfering chromogens other than creatinine, e.g. pyruvic acid. Moreover, since the non-creatinine chromogen is reported to have an inhibiting effect on colour development (Lauson, 1951) it may not be strictly correct to subtract the non-creatinine chromogen content determined with the NC-bacteria from the total chromogen content in order

to obtain the true creatinine content. With 'acid' filtrates, however, the NC-bacteria give apparently anomalous results.

It is concluded that the use of Lloyd's reagent with procedures which give 'acid' filtrates of plasma or serum, or with diluted urine, provides, at present, the most satisfactory method for the determination of the creatinine content of these fluids.

Recommended procedure for determination of creatinine in serum or urine using Lloyd's reagent

To serum (2 vol.) are added water (3 vol.), sodium tungstate (1 vol.) and sulphuric acid (2 vol.). After thorough mixing the mixture is allowed to stand for 30 min., and then filtered through a Whatman no. 1 filter paper.

Filtrate (5·0 ml.) and oxalic acid (0·5 ml.) are added to a conical centrifuge tube containing 98–102 mg. Lloyd's reagent previously tested for efficiency. Oxalic acid is essential with standard creatinine solutions and diluted urines. It is probably not necessary with 'acid' filtrates as prepared by the above method since pH is already sufficiently low. The tube is stoppered and shaken intermittently for 10 min. The tube is then centrifuged at high speed for 10 min. The supernatant fluid is decanted and the tube containing the packed sediment inverted over a filter paper until thoroughy drained.

A water blank and two standard creatinine solutions (200 μg./100 ml. and 400 μg./100 ml.) are included with each batch and are treated as filtrate. Diluted urine is treated likewise.

Alkaline picrate is prepared immediately before use by adding 5·5 ml. sodium hydroxide to 27·5 ml. picric acid and making the volume up to 100 ml. with water.

Alkaline picrate (7·5 ml.) is added to the conical centrifuge tube and the packed sediment loosened with a glass rod. The tubes are stoppered, shaken intermittently for 10 min., and then centrifuged at high speed for 10 min. The tubes are then placed in a water bath at 20° until the contents attain this temperature.

The optical densities of the supernatant fluid are measured in the Unicam SP 500 or Unicam SP 600 spectrophotometer at a wavelength of 520 mμ. using 20 mm. cells. All optical densities are read against air. A calibration curve is constructed from the readings obtained from the standard creatinine solutions.

SUMMARY

1. The application of the Jaffé reaction to the determination of creatinine in serum, plasma and urine has been examined.

2. The conditions under which the colour is developed must be carefully controlled. The effect of pH is of particular importance.

3. Both the 'apparent' creatinine content of serum and the recoveries of creatinine added to serum are influenced by the method of protein precipitation.

4. The use of the NC-bacteria or of Lloyd's reagent increases the specificity of the determination.

5. The methods using NC-bacteria or Lloyd's reagent indicate that the non-creatinine chromogen constitutes 20 and 5% of the total chromogen present in serum filtrates and diluted urines, respectively.

6. It is concluded that the use of Lloyd's reagent with procedures which give 'acid' filtrates of plasma or serum (pH < 2·5), or with diluted urine, provides, at present, the most satisfactory method for the determination of the creatinine content of these fluids.

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Further Observations on Factors Influencing the Utilization of Citrate by Yeast

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Evidence presented previously (Foulkes, 1951, 1953) showed oxidation of citrate by particulate enzyme systems from *Saccharomyces cerevisiae* to be activated by Mg^{2+} ions and a dialysable factor, citrate oxidation factor (COF), present in boiled yeast juice; this factor behaved as a weak base toward anionic exchange resins and its nature has now been further investigated.

METHODS

Preparation of aqueous extracts of yeast (A). Extracts were prepared as follows: weighed quantities (300 g.) of fresh whole yeast (Distillers' Co. Ltd., 20–21 St James Square, S.W. 1) washed twice with 0·85% (w/v) saline, were suspended in water (1:1) and heated to boiling for 5 min. The debris was removed by centrifuging at 200 *g* and washed once with water. The combined supernatant and washings were concentrated at pH 7 by freeze-drying, in the presence of phosphate buffer (50 ml., 0·1 M, $Na_2HPO_4 + KH_2PO_4$, 13:7). Change of pH during concentration of unbuffered preparations reduced the activity of the product in stimulating citrate oxidation. The active concentrate (*A*) was a yellow gum containing uracil, citric acid, nucleotides, amino acids and ammonium salts; and for the purposes of assay it was dissolved in 20 ml. of water.

Preparation of yeast enzymes. Washed yeast, 5–6 g., was fragmented by being shaken with 0·9% (w/v) KCl (4 ml.) as described by Foulkes (1951). The cellular debris was separated by centrifuging at 1200 *g*. The enzyme preparation was free from citrates and ammonium salts after dialysis in cellophan for 4·5 hr.

Assay of factors stimulating citrate oxidation. The same assay system was employed as described by Foulkes (1953) except that $MgCl_2$ was replaced by $MgSO_4$ (0·1 ml., 0·5%, w/v). Incubation was continued for 40 min. at 37° and the pH value determined before the addition of trichloroacetic acid or ethanol.

Analytical methods. The centrifuged denatured incubation mixture (0·2 ml. or 0·4 ml. as required) was used for citrate analysis (Pucher, Sherman & Vickery, 1936; Buffa & Peters, 1950). The ammonium ion concentration was determined by distillation of the liberated ammonia at pH 8·6.

RESULTS

Activity of aqueous extracts of yeast

Preliminary experiments confirmed that the enzyme preparation was able to oxidize citrate (6 μmoles/40 min.) and that this value was increased (8·9 μmoles/40 min.) by the addition of the aqueous extract *A* described above and by the fractionated product (*V*.280) described by Foulkes (1951, 1953). Chromatographic analysis showed that the stimulatory concentrate *A* contained several amino acids, uracil, nucleotides and ammonium salts. The influence of the following compounds on citrate oxidation was therefore examined severally: L-glutamic acid, L-glutamine, L-asparagine, DL-alanine, nicotinamide (slight activity), glycine, L-tyrosine, DL-aspartic acid, D-glucosamine, urea (inactive). The only compounds in our experiments