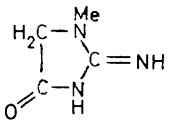
The Jaffé Reaction. Part II.[1] A Kinetic Study of the Janovsky Complexes formed from Creatinine (2-Imino-1-methylimazolidin-4-one) and Acetone

By **Anthony R. Butler**, Department of Chemistry, The University, St. Andrews, Fife

Creatinine (2-imino-1-methylimazolidin-4-one) reacts with an alkaline solution of sodium picrate to form a Janovsky complex (the Jaffé reaction). Kinetic studies show that the mechanism at 25° is rate-determining attack of the creatinine anion on picrate. At 35° the rate-determining step may be proton abstraction. Acetone also forms a Janovsky complex with picrate by the same mechanism. Both complexes react further, probably to form 2 : 1 adducts. Reaction between creatinine and an alkaline solution of trinitrobenzene is very fast. The use of the Jaffé reaction for the quantitative estimation of creatinine is discussed critically.

In 1886 Jaffé[2] reported that addition of creatinine (2-imino-1-methylimazolidin-4-one) (I) to an alkaline solution of sodium picrate results in formation of a red colour. This reaction was adapted by Folin[3] as a method for the quantitative estimation of creatinine. This is a matter of some importance as creatinine is produced endogenously by muscle activity and increased creatinine concentration in the plasma indicates renal failure. This method is still the normal one for creatinine estimation[4] but, in spite of its widespread use over many years, little is known about its chemistry. In Part I[1] evidence was



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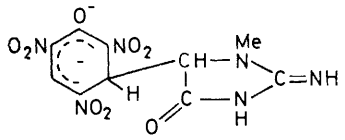


(II)

presented to show that the colour is due to the formation of the Janovsky[5] complex (II). In this paper, a detailed kinetic study of the reaction is described. Some data on this subject have been reported by Bartels and Cikes[6] but the work is not complete enough for a detailed analysis of the reaction.

RESULTS AND DISCUSSION

Addition of picric acid to an alkaline solution containing an excess of creatinine results in the spectral changes shown in Figure 1. The rate of reaction may be determined, therefore, by monitoring the increase in absorbance at 483 nm. There is a tight isosbestic point at 374 nm, which indicates that a single reaction is occurring. Under these conditions formation of the complex is a first-order process. The variation of k_{obs} with creatinine concentration is given in Table 1. Values of k_{calc} are calculated assuming that the reaction is first order in creatinine, and the good agreement with the experimentally observed values (k_{obs}) indicates that this assumption is correct.

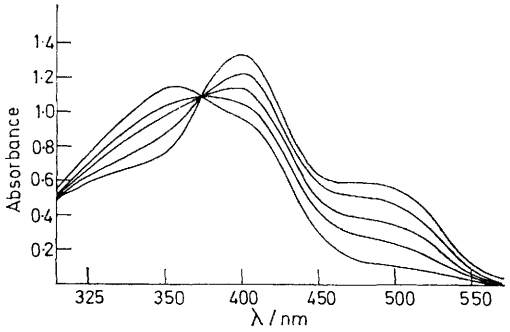


FIGURE 1 Spectral changes accompanying the reaction of creatinine and alkaline picrate. Spectra taken 0·5, 2, 3·5, 5·5, and 9 min after mixing: [picrate] = $1·73 \times 10^{-4}$M; [creatinine] = $5·20 \times 10^{-3}$M; [NaOH] = 0·080M

TABLE 1

Variation of k_{obs} with creatinine concentration in the Jaffé reaction at 25°

[picrate]$_0$ = $2·88 \times 10^{-4}$M; [NaOH] = 0·10M

10^3[creatinine]/M	1·34	2·69	4·03	6·72	13·4	20·2
$10^3 k_{obs}$/s^{-1}	1·35	2·62	3·75	6·11	13·4	18·2
$10^3 k^*_{calc}$/s^{-1}	1·25	2·50	3·75	6·25	11·6	18·8

* Assuming k_{obs} = 0·93[creatinine].

It is also possible to determine the rate of complex formation with picrate in excess, rather than creatinine.

[1] Part I, A. R. Butler, *Clinica Chim. Acta*, 1975, **59**, 227.
[2] M. Jaffé, *Z. physiol. Chem.*, 1886, **10**, 391.
[3] O. Folin, *Z. physiol. Chem.*, 1904, **41**, 223.
[4] I. D. P. Wootton, ' Microanalysis in Medical Biochemistry,' Churchill Livingstone, 1974, p. 78.
[5] J. V. Janovsky and L. Erb, *Ber.*, 1886, **19**, 2155.
[6] H. Bartels and M. Cikes, *Clinica Chim. Acta*, 1969, **26**, 1.

The spectral changes are different but the rate may be determined by observation of the absorbance changes at 530 nm. The variation of k_{obs} with picrate concentration at two hydroxide concentrations is given in Table 2 and the results show that the reaction is first

TABLE 2

Variation of k_{obs} with picrate concentration in the Jaffé reaction at 25°

$[creatinine]_0 = 1{\cdot}34 \times 10^{-4}$M; [NaOH] = 0·10M

10^3[picrate]/M	2·88	5·76	8·64	11·5	14·4	17·3	20·2	23·0
$10^3k_{obs}/s^{-1}$	0·87	1·42	1·81	2·58	3·45	4·26	4·74	5·45
$10^3k^*_{calc}/s^{-1}$	0·69	1·38	2·07	2·76	3·46	4·15	4·85	5·52

* Assuming $k_{obs} = 0{\cdot}24$[picrate].

$[creatinine]_0 = 1.34 \times 10^{-4}$M; [NaOH] = 0.30M

10^3[picrate]/M	2·88	5·76	8·64	11·5	14·4	17·3
$10^3k_{obs}/s^{-1}$	2·27	4·53	6·12	8·06	9·84	11·0
$10^3k\dagger_{calc}/s^{-1}$	1·99	3·97	5·96	7·94	9·93	11·9

† Assuming $k_{obs} = 0{\cdot}69$[picrate].

TABLE 3

Variation of k_{obs} with [NaOH] in the Jaffé reaction at 25°

[picrate] = $1{\cdot}15 \times 10^{-2}$M; $[creatinine]_0 = 1{\cdot}30 \times 10^{-4}$M

[NaOH]/M	0·10	0·20	0·30	0·40	0·50
$10^3k_{obs}/s^{-1}$	2·67	5·11	7·97	10·1	12·1
$10^3k^*_{calc}/s^{-1}$	2·6	5·2	7·8	10·4	13·0

* Assuming $k_{obs} = 0{\cdot}026$[NaOH].

order in picrate. The only remaining variable is hydroxide and, as the figures in Table 3 show, there is also a first-order dependence here.

The results so far indicate a rate equation for complex formation of the form (1). The values of k obtained

$$\text{rate} = k[\text{creatinine}][\text{picrate}][OH^-] \qquad (1)$$

from the results in Tables 2 and 3 are 2·4, 2·3, and 2·3 l^2 mol^{-2} s^{-1} and, in all these cases, picrate is in excess. All these results are consistent with the mechanism shown in Scheme 1. The rate determining step is attack by the anion of creatinine on picrate (k_2) and therefore, the over-all rate equation is (2). This is exactly the form of the experimentally determined rate equation.

$$\text{rate} = k_2k_1/k_{-1}[\text{creatinine}][OH^-][\text{picrate}] \qquad (2)$$

A problem arises when the data in Table 1 are considered for, when creatinine is in excess, k is 9·3 l^2 mol^{-2} s^{-1}. This indicates that under these conditions there must be some slight change of mechanism. The matter was not investigated further and the explanation of such a change is not obvious. In the clinical estimation of creatinine, picrate is, of course, present in excess so that the data from Tables 2 and 3 are of greater importance in elucidating the mechanism of the Jaffé reaction. The postulated mechanism in Scheme 1 correctly reproduces the observations under these conditions.

TABLE 4

Variation of k_{obs} with creatinine concentration in the Jaffé reaction at 35°

$[picrate]_0 = 2{\cdot}88 \times 10^{-4}$M; [NaOH] = 0·10M

10^3[creatinine]/M	1·30	2·60	3·90	5·20	6·50
$10^3k_{obs}/s^{-1}$	2·21	4·22	5·83	7·99	9·97
$10^3k^*_{calc}/s^{-1}$	2·05	4·10	6·16	8·21	10·3

* Assuming $k_{obs} = 1{\cdot}58$[creatinine].

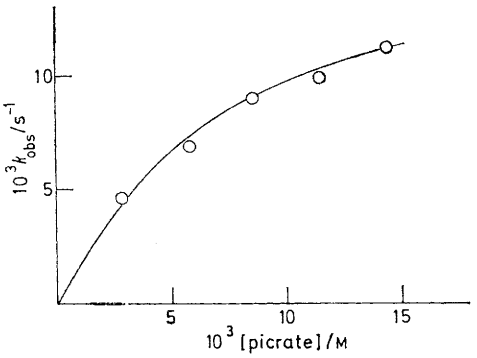
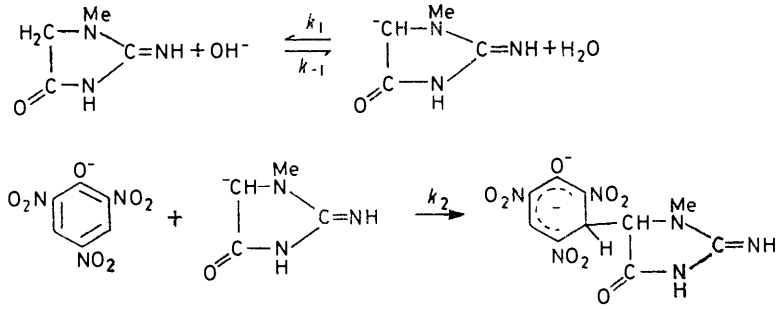


FIGURE 2 Variation of k_{obs} with picrate concentration in the Jaffé reaction at 35°: $[creatinine]_0 = 1{\cdot}30 \times 10^{-4}$M; [NaOH] = 0·30M

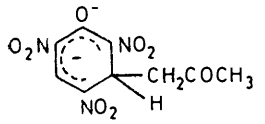
A study of the kinetics of the reaction at 35° showed a change in the mechanism. If an excess of creatinine is present then the reaction is again first order in creatinine (Table 4). However, with picrate in excess the relationship between picrate concentration and the observed rate constant is not linear (Figure 2). This indicates that, at low picrate concentration, the second reaction (k_2) is still the slow step but, as the picrate concentration is increased, this ceases to be the case, and the rate of proton removal (k_1) becomes rate determining. This situation is the same as that observed in the halogenation of acetone.[7]



SCHEME 1

[7] R. P. Bell and K. Yates, *J. Chem. Soc.*, 1962, 1931.

In the Jaffé reaction the reactive part of the creatinine molecule is the methylene group, activated by the neighbouring carbonyl group, and the other functional groups present in the molecule appear to play no part. Therefore, a similar reaction should occur with any compound containing an activated methylene group. The simplest such compound is, of course, acetone and its reaction with alkaline picrate has been examined. Addition of acetone to the yellow solution of picrate results in darkening and from this solution Kabeya *et al.*[8] isolated the disodium salt of the Janovsky complex (III). This is



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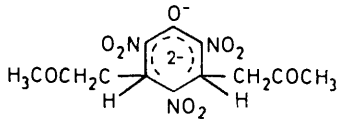

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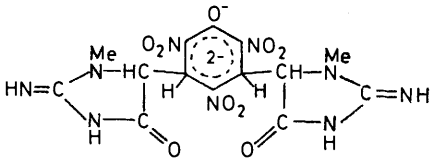


(V)

exactly parallel to the proposed reaction with creatinine. An examination of the spectral changes accompanying this reaction show that its rate may be determined by monitoring the change in absorbance at 510 nm and, with acetone in large excess, the reaction was found to be of the first order. The variation of k_{obs} with acetone concentration is shown in Figure 3. The final absorbance at

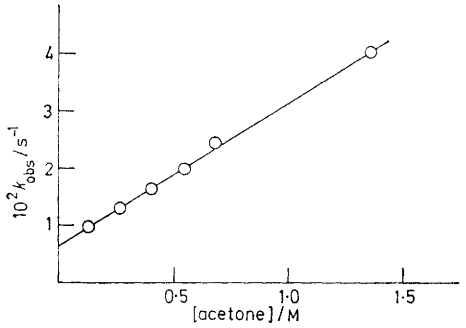
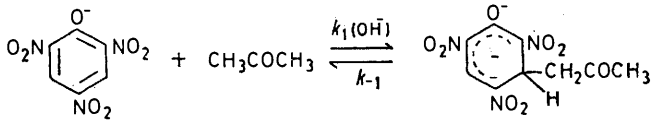


FIGURE 3 Variation of k_{obs} with acetone concentration in its reaction with alkaline picrate at 25°: $[picrate]_0 = 2{\cdot}88 \times 10^{-3}$M; [NaOH] = 0·30M

510 nm was found to vary with the acetone concentration and this observation, coupled with the intercept of the curve shown in Figure 3, indicates that formation of (III) is an equilibrium process (Scheme 2) and that, with this range of acetone concentrations, formation must be incomplete, *i.e.* the value of K, the equilibrium constant for complex formation, is small. K is defined as [complex]/[picrate][ketone] and depends upon $[OH^-]$, but all the values quoted are at 0·1M-NaOH. With creatinine, K is large (4 800 l mol^{-1}) [1] and conversion to



SCHEME 2

the complex was, in all the kinetic studies reported, essentially complete. The value of K for the acetone–picrate reaction may be calculated in two ways. For the mechanism in Scheme 2 the rate of approach to the equilibrium position, if acetone is in excess, is governed by expression (3).[9] Therefore, the slope of the curve in

$$k_{obs} = k_1[\text{acetone}] + k_{-1} \quad (3)$$

Figure 3 is k_1 and the intercept is k_{-1}. The equilibrium constant K is k_1/k_{-1} and the value obtained is 3·6 l mol^{-1}. The value of K may also be obtained from the variation of final absorbance at 510 nm with acetone concentration.[10] If these data are plotted in the form of a Benesi–Hildebrand[11] plot then the value of K obtained is 1·8 l mol^{-1}. The former is probably the better value but, considering the inaccuracies of the Benesi–Hildebrand treatment,[12] the agreement is good.

The variation of k_{obs} with hydroxide concentration is given in Table 5 and, from the agreement of k_{obs} and k_{calc},

TABLE 5

Variation of k_{obs} with hydroxide concentration for the reaction of acetone with alkaline picrate at 25°

$[picrate]_0 = 5{\cdot}76 \times 10^{-3}$M; [acetone] = 0·136M

[NaOH]/M	0·10	0·20	0·30	0·40	0·50
$10^3 k_{obs}/s^{-1}$	3·98	8·82	12·9	16·7	20·2
$10^3 k^*_{calc}/s^{-1}$	4·20	8·40	12·6	16·8	21·0

* Assuming $k_{obs} = 0{\cdot}042[OH^-]$.

it is evident that the reaction is first order in hydroxide. The results obtained show that the mechanism is rate-determining attack of the acetone anion on picrate. The same mechanism was proposed by Obi *et al.*[13] for the reaction of acetone with trinitrobenzene in alkaline solution, which also results in the formation of a Janovsky complex. The similarity between the reaction of acetone and of creatinine with alkaline picrate is further evidence in support of the mechanism proposed for the latter reaction. The value of K for the creatinine reaction is much higher than that for acetone as the pK_a of creatinine [1,14] is much lower than that of acetone,[15] *i.e.* it is a stronger acid.

In none of the experiments described has the ionic strength been kept constant, and it is important to see if this has any significant effect on the kinetics. The

[8] T. Kabeya, K. Kohashi, and Y. Ohkura, *Chem. Pharm. Bull.* (*Japan*), 1974, **22**, 711.
[9] C. F. Bernasconi and R. G. Bergstrom, *J. Amer. Chem. Soc.*, 1973, **95**, 3603.
[10] V. Gold and C. H. Rochester, *J. Chem. Soc.*, 1964, 1692.
[11] H. A. Benesi and J. H. Hildebrand, *J. Amer. Chem. Soc.*, 1949, **71**, 2703.
[12] E. H. Lane, S. D. Christian, and J. D. Childs, *J. Amer. Chem. Soc.*, 1974, **96**, 38.
[13] N. Obi, H. Kakizaki, and M. Kimura, *Chem. Pharm. Bull.* (*Japan*), 1973, **21**, 235.
[14] A. Wollenberger, *Acta Chem. Scand.*, 1953, **7**, 445.
[15] R. P. Bell and P. W. Smith, *J. Chem. Soc.* (*B*), 1966, 241.

effect of the addition of sodium perchlorate on the rate of acetone with alkaline picrate is shown in Table 6. The

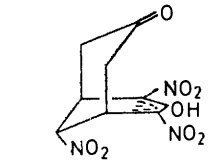
TABLE 6

Salt effect in the reaction of acetone with alkaline picrate at 25°

$[picrate]_0 = 5{\cdot}76 \times 10^{-3}$M; [acetone] = 0·136M

$[NaClO_4]$/M	0	0·111	0·207	0·413
$10^3k_{obs}/s^{-1}$	13·0	13·6	13·3	14.0

figures show that the salt effect is very small and the fact that the ionic strength varies from run to run has little effect on the kinetics.



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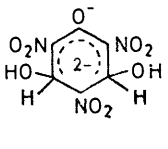

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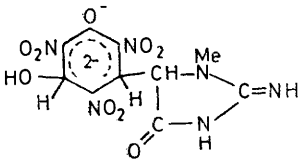

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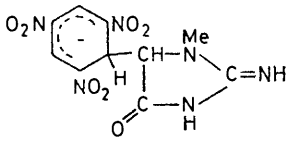

(IX)

If a reaction mixture containing an excess of acetone or creatinine is allowed to stand the colour fades during several hours. Fading is a common phenomenon with Meisenheimer complexes and may result in the release of nitrite in a process catalysed by light.[16] In the present case, no nitrite could be detected and the fading is insensitive to light. Therefore, the most likely course for the reactions is formation of the 2 : 1 adducts (IV) and (V). There is the alternative of the bicyclic compound (VI)[17] in the case of acetone. Such 2 : 1 adducts are known to be formed between picrate and hydroxide,[18] and picrate and sulphite.[19] The final spectrum, in the region 300—600 nm, obtained with both creatinine and acetone is a single peak (maxima at 391 and 394 nm respectively), while that of (VII) is also a single peak (maximum 390 nm). The similarity of spectra does suggest the same structure for all three. Bicyclic compounds, similar to (VI), have been isolated from the products of reaction of acetone and polynitro-compounds,[20] but the reaction conditions were different (acetone as solvent and triethylamine as base) and the 2 : 1 adduct is favoured in this case. The fact that creatinine gives a spectrally similar product, and cannot form a bicyclic compound, supports this designation.

Spectral changes accompanying the fading of the creatinine–picrate complex (II) have been examined. The shoulder in the region 460—500 nm disappears and there is a slight shift in the maximum to 391 nm. There is a tight isosbestic point at 360 nm and the reaction rate was obtained by monitoring the absorbance at 480 nm. The effect of creatinine concentration on the rate of this reaction is given in Table 7, and these data show

TABLE 7

Effect of creatinine concentration on k_{obs} for the fading of the creatinine–picrate complex

$[picrate] = 2{\cdot}88 \times 10^{-4}$M; [NaOH] = 0·10M

10^2[creatinine]/M	1·30	1·95	2·60	3·25	3·90	4·55
$10^3k_{obs}/s^{-1}$	2·19	3·23	4·28	5·24	6·75	7·73
$10^3k^*_{calc}/s^{-1}$	2·19	3·30	4·39	5·49	6·59	7·69

* Assuming $k_{obs} = 0{\cdot}17$ [creatinine].

that the reaction is first order in creatinine, which is consistent with the reaction being formation of a 2 : 1 adduct. The effect of varying the hydroxide concentration is more complex and the results are shown in Figure 4. This behaviour is difficult to explain. One possible explanation is that at high hydroxide concentration the 2 : 1 adduct (VIII) forms and so the rate is reduced as there is less of (II) available for reaction with a second molecule of creatinine.

Picric acid has two important functional groups, the nitro- and the hydroxy-groups, and, in the search for an improved reagent for creatinine estimation, it is relevant to know if both of these are important in the formation of coloured species. Trinitrobenzene, which lacks the

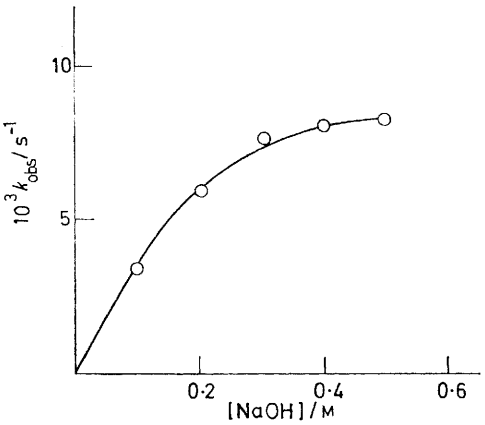

FIGURE 4 Variation of k_{obs} with hydroxide concentration for the fading of the Janovsky complex from creatinine and picrate: [creatinine] = $1{\cdot}95 \times 10^{-2}$M; $[picrate]_0 = 2{\cdot}88 \times 10^{-4}$M

hydroxy-group, reacts with hydroxide to give a complex with an intense red colour with absorbance maxima at 440 and 500 nm.[21] Addition of creatinine results in an increase in absorbance and the spectrum of the product is a single, broad peak with a maximum at 475 nm. It is reasonable to assume that this is due to complex (IX)

[16] V. Gold and C. H. Rochester, *J. Chem. Soc.* (*B*), 1964, 1704, 1710.

[17] T. Kabeya, K. Kohashi, Y. Ohkura, and T. Momose, *Chem. Pharm. Bull.* (*Japan*), 1973, **21**, 2168.

[18] V. Gold and C. H. Rochester, *J. Chem. Soc.*, 1964, 1722.

[19] M. R. Crampton and M. El-Ghariani, *J. Chem. Soc.* (*B*), 1969, 330.

[20] R. Foster and C. A. Fyfe, *J. Chem. Soc.* (*B*), 1966, 53; M. J. Strauss and H. Schran, *J. Amer. Chem. Soc.*, 1969, **91**, 3974; M. I. Foreman, R. Foster, and M. J. Strauss, *J. Chem. Soc.* (*C*), 1969, 2112; M. J. Strauss, T. C. Jensen, H. Schran, and K. O'Connor, *J. Org. Chem.*, 1972, **35**, 383.

[21] C. Bernasconi, *J. Amer. Chem. Soc.*, 1970, **92**, 4682.

and its rate of formation at 0·10M-NaOH and $6·7 \times 10^{-3}$M-creatinine was measured by stopped-flow spectrophotometry. The half-life for the reaction is *ca.* 2 s and is, therefore, over 1000 times faster than that with picrate. This difference must be due to the repulsion experienced by the creatinine anion because of the ionised hydroxy-group in picrate. In spite of the more suitable spectral changes and the increased speed of reaction, trinitrobenzene is not a suitable reagent for creatinine estimation as its solubility in water is too low. For the test to work the reagent must be in an excess over the creatinine. It may be concluded, therefore, that the role of the hydroxy-group is to make the reagent water soluble. However, its presence does produce an intense absorption in the visible spectrum and addition of creatinine, when picrate is in excess, merely results in a broadening of this peak and absorbance measurements have to be made at a wavelength which is on a rapidly rising part of the curve, with the consequent inaccuracies. With an ideal reagent, addition of creatinine would result in the formation of an entirely new absorbance peak. A number of compounds are being examined in the light of what has been learned from this mechanistic study.

The use of the Jaffé reaction for the estimation of creatinine may be criticised on other grounds and, in view of its medical importance, it is of value to consider the chemistry involved in the reported deficiencies of the test. No support has been found for the view expressed by Woolf *et al.*[22] that the reaction is affected by the degree of agitation. The concentration of hydroxide is, on the other hand, of obvious importance. High concentrations speed up the reaction which, when many samples are being tested, is of value but if the concentration is made too high then complex (VII) forms and, because of its intense absorption, reduces the absorbance change produced on addition of creatinine. The use of low hydroxide concentration has been recommended previously.[23] The main fault with the use of the Jaffé reaction is its lack of specificity.[24] Most of the substances which interfere are ketones or amines, the latter being the more important group. Addition of most amines to alkaline picrate results in immediate darkening, possibly due to the formation of charge transfer complexes.[25] This interference is inevitable if a polynitro-compound is used as the reagent and, at the moment, the best means of separation of creatinine is column chromatography.[26]

EXPERIMENTAL

Materials.—Creatinine was used without purification. All the other reagents were AnalaR grade.

Kinetic Method.—Standard solutions of the reactants were prepared and thermostatted in a constant temperature water-bath before mixing. The reaction mixture was placed in a cuvette in the thermostatted cell holder of a Unicam SP 700 spectrophotometer and the variation of absorbance with time at the wavelengths indicated was determined. As the colours faded, it was difficult to obtain a definite infinity reading and so the first-order rate constants were calculated by the Swinbourne–Kezdy[27] method. The reaction between creatinine and trinitrobenzene was examined by the use of a Canterbury stopped-flow spectrophotometer. Nitrite was tested for by means of the Geiss reaction.[28]

[4/2475 *Received*, 27*th November*, 1974]

[22] P. L. Wolf, D. Williams, T. Tsudaka, and L. Acosta, 'Methods and Techniques in Clinical Chemistry,' Wiley–Interscience, New York, 1972, p. 156.
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