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Measurement of regional cerebral blood flow with antipyrine-^{14}C in awake cats

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REIVICH, MARTIN, JANE JEHLE, LOUIS SOKOLOFF, AND SEYMOUR S. KETY. *Measurement of regional cerebral blood flow with antipyrine-^{14}C in awake cats.* J. Appl. Physiol. 27(2): 296–300. 1969.—Antipyrine-^{14}C was utilized for the quantitative measurement of regional cerebral blood flow in cats by means of an autoradiographic technique. The advantages of this technique over a previously described method are the uniformity of the brain-blood partition coefficient for antipyrine, the better resolution of the autoradiograms, the ability to make permanent ^{14}C standards, the ability to obtain adjacent sections for histological studies, and the exposure of the autoradiograms at room temperature rather than at −70 C. Errors in the determination of the tissue concentration of antipyrine-^{14}C can arise from several sources. Those due to errors in replicate optical density measurements plus variations in section thickness were found to be 5.6%. The standards used introduced only a small error since the standard deviation of replicate standards was no greater than 1.1%. Regional cerebral blood flow was measured in a series of six awake, unrestrained resting cats. The values varied from 0.21 ml/g per min in the cerebral white matter to 1.74 ml/g per min in the inferior colliculus. Arterial P_{CO_2} was 34 ± 2.9 mm Hg, P_{O_2} was 102 ± 8.4 mm Hg, pH was 7.44 ± .01, and hematocrit was 29 ± 6.6%.

autoradiography; arterial P_{CO_2}; arterial P_{O_2}; arterial pH; hematocrit; unrestrained animals

IN 1955, LANDAU ET AL. (5) described an autoradiographic technique for the determination of regional cerebral blood flow utilizing trifluoroiodomethane-^{131}I, $CF_3{}^{131}I$. This technique is an application of the principles of inert gas exchange between the blood and tissues which have been extensively reviewed (3). The inert gas, $CF_3{}^{131}I$, was dissolved in Tyrode's solution and administered intravenously over a 1-min period. During this period, the arterial concentration of the gas was determined by drawing blood through a helical glass tube in a well-type scintillation counter. At the end of the 1-min infusion, the animal was decapitated, the head frozen and cut into coronal sections about 5 mm thick on a band saw, and autoradiograms prepared from these sections which also contained ^{131}I standards embedded in them.

There are certain limitations to the original technique. $CF_3{}^{131}I$ is not commercially available and must be synthesized for each experiment. Possible errors may be introduced by volatilization of the gas from the surface of the frozen section. The use of thick sections makes it difficult to prepare adjacent sections for histological comparison. The relatively high energies of the beta radiations of the ^{131}I label lowers the resolution of the autoradiographs, and the short half-life of the isotope introduces timing problems. Finally, the solubility of $CF_3{}^{131}I$ in blood varies with hematocrit, and the partition coefficients between the various tissues and blood, therefore, vary from cat to cat. Thus, it is necessary to determine them in each animal. For these reasons, and because of certain advantages to be pointed out, the following technique was developed.

Antipyrine-^{14}C was used as the inert tracer in the present studies for several reasons. Antipyrine is freely diffusible in total body water and its distribution in the body is proportional to the water content of the various tissues (1). This suggested that the brain-blood partition coefficient might be similar for all regions of the brain. The compound is very slowly metabolized in the liver (6% per hr) so that the possible error from this source would be negligible in the 1 min duration of these studies.

The use of a ^{14}C label has several advantages. It is a pure beta-emitter of low energy (0.155 Mev) and therefore produces autoradiograms of better resolution than ^{131}I which, in addition to having a higher energy beta-emission (0.608 Mev), is also a gamma-emitter. Furthermore, the long half-life of ^{14}C enables permanent standards to be made. The long half-life also allows a smaller dose of isotope to be used since longer exposure times are possible.

The use of a nongaseous tracer obviated its possible loss from the surface of the sections thus making it possible to use a cryostat to cut 20-μ-thick sections. This allowed serial sections to be obtained so that histological studies could be undertaken along with the autoradiographic studies. Since the tracer was nongaseous, the autoradiograms could be made at room temperature instead of −70 C. Further modifications included the blood sampling technique and the use of a liquid scintillation counter for determining the ^{14}C concentration in the blood. In addition a program was written for a digital computer to perform the calculations necessary in computing the regional blood flow values.

METHODS

The technique like its predecessor, is based on the Fick principle, which for a biologically inert diffusible tracer substance may be stated as follows: the time rate of change of the amount of tracer substance within the tissue is equal to the difference between the rate at which the substance is brought to the tissue in the arterial blood and removed from it in the venous blood. It can be shown that this can be expressed mathematically as follows (4):

$$C_i(T) = \lambda k_i \int_0^T C_a e^{-k_i(T-t)}\, dt \qquad (1)$$

where $C_i(T)$ = the concentration of tracer substance in the tissue at time T; λ = the tissue-blood partition coefficient for the tracer material; k_i = the rate of blood flow per unit weight of tissue multiplied by the reciprocal of the partition coefficient for that tissue; and C_a = the concentration of tracer substance in the arterial blood. Thus, to calculate the blood flow to a given homogenous region of the brain one must know: *1*) the tissue-blood partition coefficient of the tracer substance for that region of the brain, *2*) the concentration of the tracer substance in that region of the brain at some time, T, which in these studies was 1 min after the start of the infusion, and *3*) the time course of change of arterial concentration of tracer material.

Determination of brain-blood partition coefficient. Since antipyrine is both excreted unchanged in the urine and metabolized in the

liver (1), to obtain a steady state for the determination of the brain-blood partition coefficient the arterial blood supply to the kidneys and the venous outflow from the liver was occluded by ligatures in a series of 15 rats. Antipyrine-^{14}C was then injected intravenously. At the end of 1 hr, when equilibrium was reached, a blood sample was obtained by cardiac puncture and the animal was decapitated and the brain removed and frozen in a mixture of acetone and Dry Ice at −30 C. The frozen brain was then cut in half sagittally. From one-half of the brain 20-μ sections were cut in a cryostat to be used for the preparation of autoradiograms as described below. From the other half of the brain samples of white and gray matter were obtained in which the concentration of antipyrine-^{14}C was determined directly by counting techniques. The latter samples were weighed, lyophilized in a freeze-dry apparatus, dissolved in Hyamine, and counted in a toluene-ethanol phosphor solution in a liquid scintillation counter. The blood samples were counted as described below.

The means and standard errors of 17 determinations of the brain-blood partition coefficient in gray and white matter were 1.00 ± 0.026 and 0.97 ± 0.026, respectively. There was no significant difference between these values ($0.4 > P > 0.3$). Therefore, the average, 0.99 was used as the value of the brain-blood partition coefficient. There was no difference for the value of the partition coefficient among various white or gray structures throughout the entire brain, as demonstrated by the autoradiograms which showed a homogenous density throughout (Fig. 1).

Collecting and counting of blood samples. Arterial blood samples were obtained via a PE-50 polyethylene catheter placed in the femoral artery of the cat and advanced into the descending aorta. An automatic sampling device consisting of a rotating stopcock arrangement shown in Fig. 2 allowed us to collect 0.04-ml samples every 6 sec. These were collected consecutively as individual spots of blood on a strip of no. 3 Whatman filter paper. Each spot was cut out and placed in a counting vial containing 10 ml of naphthalene-dioxane phosphor solution and 1 ml of water. The vials were placed on a mechanical shaker for 24 hr and then counted in a liquid scintillation counter. This period of time is more than adequate since studies of the time course of elution of the antipyrine-^{14}C from the filter paper in 11 samples revealed a time constant whose mean and standard error were 6.2 ± 0.2 hr even without shaking. Therefore, in 24 hr at least 98% of the antipyrine-^{14}C is eluted. The process of elution probably is even faster when a shaker is used. Further studies showed that the presence of the filter paper itself in the counting vial did not cause quenching. However, the blood spot did cause a slight amount (4%) of quenching. Therefore, standards were made up containing a known amount of antipyrine-^{14}C in the blood spot and were counted with the unknown samples. Alternatively, an internal ^{14}C standard can be used.

Removal and sectioning of brain. At the appropriate time, the animals were killed by the rapid intravenous injection of saturated KCl via a catheter placed far up the inferior vena cava. Blood pressure dropped within 5 sec to levels inadequate to sustain any cerebral perfusion. The brains were than rapidly removed and frozen in acetone-Dry Ice at −30 C. Studies have shown that there is no detectable loss of resolution in the autoradiograms by removing the brain first and then freezing it rather than freezing the whole head and then removing the frozen brain. The frozen brain was then cut grossly into two coronal sections and frozen to microtome chucks with 5% gum tragacanth. Twenty-micron sections were then cut in a cryostat at −19 C. Every 25th section was taken for autoradiograms and every 26th for histology. The sections were picked up from the knife with a coverslip at room temperature and immediately placed on a hot plate at 60 C (7). The sections dried within 2–3 sec. Studies were performed comparing the autoradiograms obtained by this method with those obtained from sections which were kept frozen at all times. There was no apparent loss of resolution with this quick-drying method. The coverslip containing the dried section was then affixed to a glass slide with Krylon (Krylon no. 1303, Krylon, Inc., Norristown, Pa.) so that the section was exposed. In a dark room, sections were placed face down on Kodak single-emulsion medical X-ray film in X-ray cassettes. A set of standards containing known concentrations of antipyrine-^{14}C was also placed on each film so that a calibration curve could be obtained for each film relating optical density to ^{14}C concentration. These standards corrected for any differences in exposure and developing that occurred between films. In Fig. 3 are illustrated some autoradiograms obtained with this technique.

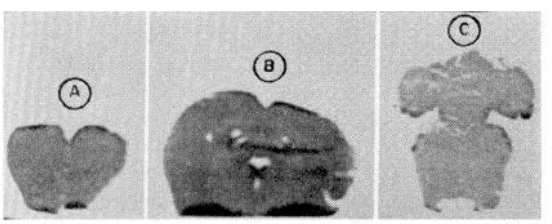

FIG. 1. Autoradiograms prepared from coronal sections of a rat brain 1 hr after the intravenous injection of antipyrine-^{14}C. *A:* through the anterior frontal lobes; *B:* through the posterior frontal lobes at the level of the optic chiasm; *C:* through the cerebellum and brain stem. Dark areas at the edges in *A* and *B* are artifacts due to folding over of the section.

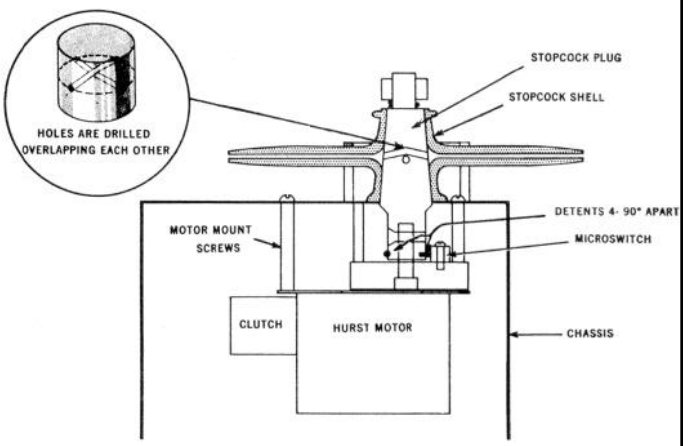

FIG. 2. Motor-driven stopcock with two equal-volume noncommunicating channels for blood sample collection. Microswitch arrangement controls the periodic energizing of the motor by a control circuit consisting of a simple single-shot multivibrator driven by a very stable clock. A source of compressed air is used to empty the blood sample from the bore of the stopcock onto a moving strip of no. 3 Whatman filter paper.

Preparation of autoradiographic standards. The standards which were placed on each film were made from the brains of rats that had been prepared as described above for the determination of the brain-blood partition coefficient. Rat-brain standards were used because less isotope was required to prepare them and the absorption characteristics should be very similar to that of brain from other species. In a series of 15 animals, various amounts of antipyrine-^{14}C were injected intravenously, so that the concentration in the brain at the end of 1 hr varied from 25 to 2,100 mμc/g. The brains were removed and frozen 1 hr after the intravenous injection and cut in half sagittally. From one-half brain, 20-μ sections were cut and used as standards, and from the other half samples of brain tissue were taken and assayed for antipyrine-^{14}C concentration as described above. The standards prepared in this manner are good for only 4–6 weeks since, being unfixed tissue, they begin to deteriorate and changes in the antipyrine-^{14}C concentration occur. Therefore, permanent standards were made of plastic containing antipyrine-^{14}C which were calibrated against freshly pre-

pared rat-brain standards to correct for differences in the absorption characteristics between plastic and brain tissue. The values of the plastic standards ranged from 190 to 1,990 mμc/g in units equivalent to brain concentration. A blank was also placed on each film which consisted of a plastic standard which contained no radioactivity.

Errors in determination of tissue concentration of antipyrine-^{14}C. Several factors may contribute to errors in the determination of the tissue concentration of antipyrine-^{14}C from autoradiograms. Since the sections are less than infinite thickness for ^{14}C, variation in the thickness of each 20-μ section will produce apparent changes in antipyrine-^{14}C concentration. Also, the variability in duplicate readings of the optical density of the autoradiograms contributes an error. The magnitude of the error from these two sources was determined by making multiple readings on a series of 16 sections cut from one of the rat-brain standards. The variation within sections was 1.9% of the mean optical density value, and the variation between sections was 5.6%. The variation within sections is probably due mainly to errors in making duplicate optical density measurements. The variation between sections includes this error plus the error due to variations in the section thickness. All optical density measurements were made with a Photovolt densitometer model 501 A.

Variability in replicate samples of the standards is another source of error. This was reduced by exposing all the standards on X-ray film and reading their optical density. The most divergent standards in each group were discarded so that the resulting standard deviation of each mean value was no greater than 1.1% of that value.

Catheter smearing correction. The theoretical basis of the method requires that the shape of the curve of arterial concentration of isotope from time zero to the time the tissue concentration is measured be known.

The arterial blood samples are drawn through a PE-50 catheter which can cause distortion of the shape of the arterial curve. Studies were therefore done to determine the smearing characteristics of the catheter and sampling system. A step function change in antipyrine-^{14}C was introduced into the catheter as follows. A section of catheter, approximately 15 cm longer than the length to be investigated was attached to the sampler with the other end in a beaker of blood. At a point 15 cm from the end, the catheter was placed under the surface of blood containing antipyrine-^{14}C. As the nonradioactive blood was being pulled through the catheter, the radioactive blood was introduced without any intervening air bubble by cutting the catheter at the 15-cm point. The results of such an experiment are shown in Fig. 4. Similar studies were performed with blood of varying hematocrit since this was found to alter the smearing characteristics of the sampling system. The resulting curves could all be described by an equation of the form (see APPENDIX):

$$f_o(t) = 1 - A_1e^{-r_1t} + A_2e^{-r_2t} \qquad (2)$$

where $f_o(t)$ represents the concentration of antipyrine-^{14}C delivered from the catheter at any time t, A_1 and A_2 are the relative sizes of the exponentials, and r_1 and r_2 are their rate constants. Table 1 shows the values for r_1 and r_2 for various hematocrits. Since these values varied so much the catheter smearing function was determined after each experiment using that animal's blood. Similar experiments with a pressurized blood-reservoir system revealed no significant change in the smearing characteristics with pressures in the range of the mean pressure of cats.

Knowledge of the response, $f_o(t)$, of the sampling system to a step function change in concentration of isotope defines the behavior of the system for any type of change in isotope concentration. We are interested in the shape of the curve defining arterial concentration of isotope, $C_a(t)$, during the *time zero* to T, since the relationship between the concentration of isotope in a particular region of the brain, $C_i(T)$, at time, T, to the blood flow to that region, k_i, is described by *equation 1* above. However, we only know the time course of arterial isotope concentration after it has been smeared through the sampling system. With *equation 1* above and Laplace transforms, it can be shown that the relationship be-

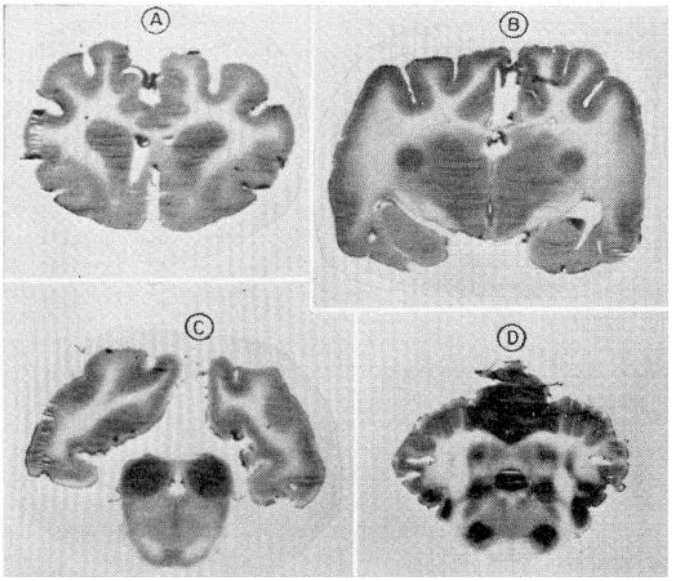

FIG. 3. Autoradiograms prepared from coronal sections of the brain of an awake unrestrained cat. *A:* at the level of the caudate nucleus, septal area, and the putamen; *B:* at the level of the lateral geniculate body, cerebral peduncle, optic tract, and hippocampus; *C:* through the inferior colliculi, aqueduct, pons, and posterior suprasylvian gyrus; *D:* through the cerebellum and medulla oblongata. The darker the region the higher is the regional blood flow.

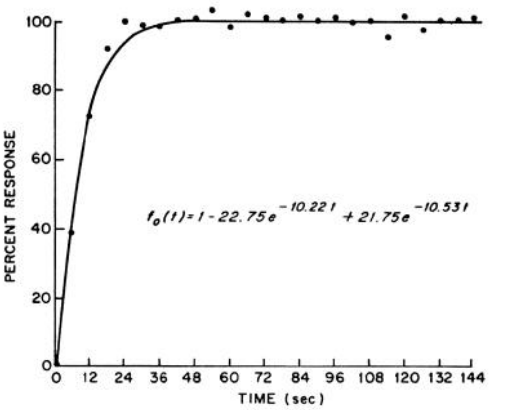

FIG. 4. Response of arterial sampling system to a step function input. Response can be described by an equation of the form illustrated, i.e., the difference of two exponentials.

TABLE 1. *Sampling system smearing characteristics at various hematocrits*

Hematocrit, %	r_1	r_2
17	7.077	7.432
25	8.002	17.45
47	2.993	6.217

tween these two time courses in operational notation is:

$$C_a(t) = \left[\frac{1}{r_1 r_2} D^2 + \frac{r_1 + r_2}{r_1 r_2} D + 1\right] c_a(t) \qquad (3)$$

where r_1 and r_2 are defined in *equation 2* above and D^2 and D are, respectively, the second and first derivative operators, and $c_a(t)$ is the smeared arterial curve obtained from the sampling system. Substituting *equation 3* into *equation 1* yields:

$$C_i(T) = \frac{\lambda_i k_i (k_i - r_1)(k_i - r_2)}{r_1 r_2} \int_0^T c_a(t)\, e^{-k_i(T-t)}\, dt - \frac{\lambda_i k_i (k_i - r_1 - r_2)}{r_1 r_2} c_a(t) + \frac{\lambda_i k_i}{r_1 r_2} D\, c_a(T) \qquad (4)$$

thus defining k_i in terms of measurable variables. (See APPENDIX for complete derivation of *eq 3* and *4* above.)

Since this equation cannot be solved explicitly for k_i a program was written for the IBM 1620 computer to calculate by means of the trapezoid rule the value of $C_i(T)$ for various values of k_i from 0 to 3.8 ml/min per g. This table was then used to find the flow for given $C_i(T)$.

RESULTS

With the technique as described above, regional cerebral blood flow was measured in six awake unrestrained resting cats. The animals were kept in a lighted box 1 x 2 ft square and 2 ft high containing a window at each end and which was ventilated from a compressed air source. Catheters had been placed in the femoral artery and vein 4–7 days before the experiment and kept open with a heparin solution. Immediately before the regional blood flow measurement, an arterial blood sample was drawn and analyzed for P_{CO_2}, P_{O_2}, pH, and hematocrit.

The determinations of pH, P_{CO_2}, and P_{O_2} were made by means of an Instrumentation Laboratory system (model 113–S2) utilizing a glass pH electrode (no. 11070) with a calomel reference electrode (no. 13077), a Severinghaus P_{CO_2} electrode, and a Clark P_{O_2} electrode. The electrodes were contained in a water bath at 37 C which was thermostatically controlled to within ±0.02 C. The electrodes were calibrated before each determination and an appropriate standard was read after each determination. National Bureau of Standards buffers were used to calibrate the pH electrode. The P_{CO_2} electrode was calibrated with gas mixtures containing known concentrations of CO_2 which had been determined with a Scholander apparatus (6). The P_{O_2} electrode was calibrated with blood samples that had been tonometered with gas mixtures of known O_2 concentrations which had been determined with a Scholander apparatus. The hematocrit was measured by means of a Guest-Weichselbaum micro hematocrit apparatus.

The values for these functions are shown in Table 2 and compared with the only other available data for awake intact cats (2). Table 3 shows the values obtained for the flow to various regions of the brain. They are compared with the values obtained with the original method as described by Landau et al. Of the 23 regions compared, in only 3 was there a significant difference between the flows obtained with these two methods. At the 0.05 level of significance, one would expect between one and two significant differences to occur by chance alone when this many comparisons are made. Therefore, there is excellent agreement between the two methods of measuring regional cerebral blood flow, and the present technique offers numerous advantages in convenience, accuracy, and amount of information obtainable.

TABLE 2. *Arterial blood constituents*

P_{CO_2}, mm Hg	P_{O_2}, mm Hg	pH	Hematocrit, %	
34±2.9	102±8.4	7.44±0.01	29±6.6	Present study
28±0.7		7.38±0.01	29±1.8	Fink and Schoolman (2)

Values are means ± SE.

TABLE 3. *Regional cerebral blood flow in awake cats*

	Landau et al. (5), 10 Cats	Present Study, 6 Cats	P Value
Superficial cerebral structures			
Cortex			
Sensory motor	1.38±0.12	1.09±0.04	
Auditory	1.30±0.05	1.22±0.11	
Visual	1.25±0.06	1.17±0.04	
Miscellaneous association	0.88±0.04	0.81±0.05	
Olfactory	0.77±0.06	0.74±0.05	
White matter	0.23±0.02	0.21±0.01	
Deep cerebral structures			
Medial geniculate body	1.22±0.04	1.43±0.11	
Lateral geniculate body	1.21±0.08	1.64±0.14*	<0.02
Caudate nucleus	1.10±0.08	1.02±0.07	
Thalamus	1.03±0.05	1.06±0.06	
Hypothalamus	0.84±0.05	0.68±0.06	
Basal ganglia and amygdala	0.75±0.03		
Amygdala		0.54±0.03	
Hippocampus	0.61±0.03	0.62±0.04	
Optic tract	0.27±0.02	0.20±0.01*	<0.05
Midbrain and pons			
Inferior colliculus	1.80±0.11	1.74±0.08	
Superior olive	1.17±0.13	1.08±0.07	
Superior colliculus	1.15±0.07	1.10±0.16	
Inferior olive		0.75±0.03	
Reticular formation	0.59±0.05	0.65±0.03	
Cerebellum and medulla			
Cerebellum			
Nuclei	0.79±0.05	0.84±0.03	
Cortex	0.69±0.04	0.83±0.03*	<0.05
White matter	0.24±0.01	0.24±0.01	
Medulla			
Vestibular nuclei	0.91±0.04	0.92±0.03	
Cochlear nuclei	0.87±0.07	0.95±0.11	
Pyramid	0.26±0.02	0.22±0.02	

Values are means ± SE, given in ml/g per min. * Significant differences. All other pairs of values not significantly different.

APPENDIX

The relationship between the true arterial time course of isotope concentration, $C_a(t)$, and this function after it has been smeared through the sampling system, $c_a(t)$, was determined by introducing a step function change in isotope concentration, $f_1(t)$, into the sampling system and determining the output function, $f_o(t)$, as described above. Empirically, it was found that the function $f_o(t)$ could be adequately described by an equation of the form:

$$f_o(t) = 1 - A_1 e^{-r_1 t} + A_2 e^{-r_2 t} \qquad (5)$$

Therefore, the transfer function, T, (i.e., that function which when convoluted on the input function produces the output function) which in the s domain is defined as:

$$T = \frac{F_o(s)}{F_1(s)} \qquad (6)$$

is described by the equation

$$T = 1 - A_1 \frac{s}{(s + r_1)} + A_2 \frac{s}{(s + r_2)} \quad (7)$$

or since $1 - A_1 + A_2 = 0$

$$T = \frac{A_1 r_1}{s + r_1} - \frac{A_2 r_2}{s + r_2} \quad (8)$$

Assuming the first derivative of $f_o(t)$ is zero at *time zero*, then

$$A_1 r_1 - A_2 r_2 = 0 \quad (9)$$

and

$$T = \frac{r_1 r_2}{s^2 + (r_1 + r_2)s + r_1 r_2} \quad (10)$$

or in the time domain

$$C_a(t) = \left[\frac{1}{r_1 r_2} D^2 + \frac{r_1 + r_2}{r_1 r_2} D + 1\right] c_a(t) \quad (11)$$

where $C_a(t)$ is the input function and $c_a(t)$ the output function. Substituting *equation 11* into *equation 1* above

$$C_i(T) = \lambda_i K_i \int_0^T \left(\frac{1}{r_1 r_2} D^2 + \frac{r_1 + r_2}{r_1 r_2} D + 1\right) c_a(t) e^{-k_i(T-t)}\, dt \quad (12)$$

which after separation into three integrals and integrating by parts produces the final solution:

$$C_i(T) = \frac{\lambda_i k_i (k_i - r_1)(k_i - r_2)}{r_1 r_2} \int_0^T c_a(t) e^{-k_i(T-t)}\, dt - \frac{\lambda_i k_i (k_i - r_1 - r_2)}{r_1 r_2} c_a(T) + \frac{\lambda_i k_i}{r_1 r_2} D c_a(T) \quad (13)$$

In testing the operator shown in *equation 11* by operating on the generalized function:

$$f(t) = 1 + Ae^{-r_1 t} + Be^{-r_2 t}$$

it can be shown that the operator converts this function to a step function.

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